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(54) Title: GENETICALLY ENGINEERED IMMUNOGLOBULINS

(57) Abstract

This invention relates to the introduction of oligopeptide epitopes of influenza virus nucleoprotein for expressing within the three dimensional fold of an immunoglobulin (Ig) molecule, thus creating molecules useful to induce specific, biologically active anti-viral immunity.

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GENETICALLY ENGINEERED IMMUNOGLOBULINS

Field of the Invention

We have developed a new method for the presentation of immunogenic epitopes to cytotoxic T lymphocytes (CTL). The new method is based on antibody antigenization, a process whereby one or several loops of an immunoglobulin molecule are re-engineered to encompass the sequence of selected portions of pathogens (virus and parasites), self antigens and tumor antigens.

The present invention may utilize in its preferred embodiments, the use of recombinant DNA technology to genetically engineer natural or synthetically-derived immunoglobulin molecules, imparting therein novel epitopes, so as to create novel entities that can be employed *in vitro* and *in vivo* in a variety of means, such as to immunize against pathogens, and for example, build tolerance to antigens. In preferred embodiments, the epitopes are inserted into the so-called heavy or light chain variable domain of a given immunoglobulin molecule. Thus, known recombinant DNA technologies come to bear in the present invention, helping create novel immunoglobulin entities that retain functionality by localizing to particular cell types mechanistically via the so-called constant domains but otherwise functionally exploited to provide a novel localization of a particular antigenic determinant or epitope.

Background of the Invention

Recombinant DNA technology has reached the point currently of being capable, in principle, of providing the methodology sufficient to identify, isolate and characterize DNA sequences, configure them for insertion into operative expression vectors and transfect those vectors variously into recombinant hosts such that those hosts are harnessed in their ability to produce the polypeptide encoded by the DNA sequence. Obviously, many variations attend the methodology associated with recombinant DNA technology, and particular means are not without inventive faculty. Nonetheless, methods are generally known in the published literature enabling requisite mental equipment for the art skilled to practice recombinant DNA technology in the production of polypeptides from a given recombinant host system.

Immunoglobulins (Igs) are the main effectors of humoral immunity, a property linked with their ability to bind antigens of various types. In view of the myriad numbers of antigens to a particular host organism, it can be appreciated that there are a like number or more of immunoglobulins that contain antigenic determinants or epitopes against particular such antigens. Immunoglobulin molecules are unique in their functionality of being capable of localizing to certain cell types, probably by means of mutual recognition of certain receptors that are located on the cell membrane. Immunoglobulins demonstrate a second general property whereby they act as endogenous modulators of the immune response. Igs and their idiotypic determinants have been used to immunize at the B- and/or T-cell level against a variety of exogenous antigens. In many cases, the immunity they evoke is comparable with that induced by the antigen itself. Although the principle underlying this phenomenon is understood, little is known about the molecular basis and the minimal structural

requirements for the immunogenicity of Igs molecules and the interaction between those regions which may be responsible for such immunogenicity and the regions that are thought to provide the localization of a given immunoglobulin molecule with a particular cell/receptor type.

In the last many years, much progress has been made in endeavors to understand the immunogenic properties, structure and genetics of immunoglobulins. See Jeske, et al., Fundamental Immunology, Paul, ed., Raven Press, New York (1984), p. 131 and Kabat, Journal Immunology *141*:525 (1988). Initially, the antigenicity of the so-called variable (V) domain of antibodies was demonstrated. Oudin, et al., Academy of Sciences D *257*:805 (1963) and Kunkel et al., Science *140*:1218 (1963). Subsequently, further research pointed out the existence of discrete areas of variability within V regions and introduced the notion of hypervariable (HV) or complementarily-determining regions (CDR). Wu, et al., J. Exp. Med. *132*:211 (1970). Many studies since have indicated that the immunogenic property of Ig molecules is determined presumably primarily by amino acid sequence contained in the CDRs. Davie, et al., Ann. Rev. Immunol. 4:147 (1986).

The basic immunoglobulin or antibody structural unit is well understood. The molecule consists of heavy and light chains held together covalently through disulfide bonds. The heavy chains are also covalently linked in a base portion via disulfide bonds and this portion is often referred to as the so-called constant region which is thought responsible for a given immunoglobulin molecule being mutually recognizable with certain sequences found at the surface of particular cells. There are five known major classes of constant regions which determine the class of the immunoglobulin molecule and are referred to as IgG, IgM, IgA, IgD and IgE. The N-terminal regions of the so-

called heavy chains branch outwardly in a pictorial sense so as to give an overall Y-shaped structure. The light chains covalently bind to the Y branches of the two heavy chains. In the regions of the Y branches of the heavy chains lies a domain of approximately 100 amino acids in length which is variable, and therefore, specific for particular antigenic epitopes incidental to that particular immunoglobulin molecule.

It is to the Y branches containing the variable domains harboring the antigenic epitopes to which the particular attention is directed as a predicate of the present invention.

Prior researchers have studied and manipulated entire CDRs of immunoglobulins, producing chimeric molecules that have reported functionality. Exemplary attention is directed to Jones, et al., Nature *321*:522 (1986) reporting on a V-region mouse-human chimeric immunoglobulin molecule. This research thus amounted to a substantially entire CDR replacement as apparently does the research reported by Verhoeyen, et al., Science *239*:1534 (1988); Riechmann, et al., Nature *332*:323 (1988); and by Morrison, Science *229*:1202 (1985). See also European Patent Application Publication No. 125023A, published 14 November 1984.

Bolstered by the successful research summarized above that resulted presumably in functional chimeric molecules, the goal of the present research was to explore further the variable region contained in the N-terminus Y branches. It was a goal of the present research to manipulate these variable regions by introduction or substitution of novel determinants or epitopes so as to create novel immunoglobulin molecules that would possibly retain the localization functionality and yet contain functional heterologous epitopes. In this manner, the novel immunoglobulin molecules hereof could be employed for use

within the organism at foreign sites, thereby imparting immunity characteristics in a novel site-directed manner. A problem facing the present researchers at that time lay in the fact that epitopes are found in a region of the Y branch. Therefore, it was difficult to envision whether any manipulation of the variable region would be possible without disrupting the interaction of heavy chain with the corresponding light chain, and if that proved inconsequential, whether the resultant molecule would retain its functionality, with respect to the novel epitope, in combination with the constant region of the basic immunoglobulin molecule. Thus, even hurdling the problem of where to experiment, it was not possible to predict whether one could successfully produce such novel, bifunctional immunoglobulin molecules.

Recognition of antigen peptides by T lymphocytes is restricted by the major histocompatibility complex (MHC) gene products and is mediated by the T-cell receptor (TCR) recognition structure.

$CD4^+$ T helper lymphocytes recognize antigen peptides presented in the context of class II MHC molecules (Unanue, Curr. Opin. Immunol. 4:63 (1992)) while $CD8^+$ cytotoxic T lymphocytes (CTL) require that antigen peptides be presented by class I MHC molecules (Braciale, Curr. Opin. Immunol. 4:59 (1992)). In both instances, antigens need to be processed into small peptides, 9 residues for peptides that bind class I molecules (Rotzschke et al., Nature 348:252 (1990)) and 13-17 residues for peptides that bind class II molecules (Chicz et al., Nature 358:764 (1992); Rudensky et al., Nature 353:622 (1991)). Antigens presented to $CD4^+$ T cells derive primarily from exogenous proteins that are processed into peptides that bind class II MHC molecules in the endocytic compartment (Unanue, Curr. Opin. Immunol. 4:63 (1992)), albeit proteins from the cytosol can also bind class II MHC molecules (Malnati et

al., Nature 357:702 (1992)). Antigens presented to CD8[+] T cells derive mostly from proteins processed in the cytosol and bind to class I MHC molecules in the endoplasmic reticulum (ER) (Braciale, Curr. Opin. Immunol. 4:59 (1992)). Prototype antigens for this type of presentation are viral proteins generated by intracellular replication of an infectious virus (Long et al., Immunol. Today 10:45 (1989)).

While cytosolic proteins are fragmented into peptides and translocated across the ER membrane by proteasomes, intracellular polypeptides that map to the class II MHC complex (Glynne et al., Nature 353:357 (1991)), endogenous proteins destined for secretion are synthesized by ribosomes attached to the rough ER. In both instances peptides from endogenous proteins complex with class I MHC molecules in a pre-Golgi compartment.

B lymphocytes are specialized antigen presenting cells (APC) (Lanzavecchia, Immunol. Today 10:157 (1989)) that express both class II and class I MHC molecules. As such, B cells constitutively present immunoglobulin peptides within the MHC molecules expressed at their surface in the context of class II molecules (Rudensky et al., Nature 359:429 (1992). However, there exists only indirect evidence that endogenous Ig peptides are presented in class I MHC molecules (Shinohara et al., Nature 336:481 (1988); Weiss et al., Cell 64:767 (1991); Yamamoto et al., Eur. J. Immunol. 17:719 (1987)) and no endogenous Ig peptide has been isolated from B cells that mediates CTL function.

It was predicted that the translated polypeptide encoded by antigenized antibody gene would follow the secretory pathway. There is large indirect evidence in favor of this assumption. First, is the demonstration that unassembled immunoglobulin μ chains accumulate in the ER where they undergo processing (Sitia et al., 1990). In the absence of

any L chain, the H chain is retained in the ER bound to the H chain-binding protein (BiP) (Bole et al., 1986). By analogy, processing of the λ2 [315] L chain and generation of an idiotype peptide presented in class II molecules was shown to occur in the ER (Weiss et al., 1989). Recently, it was demonstrated that the ER possess an enzyme necessary to cleave the leader sequence from a nascent class I molecule.

One important issue in current immunology is to be able to program the immune system towards preselected T-cell epitopes, whether these be restricted by class I or class II MHC molecules. This is significant in defensive immune responses against pathogens, and, in particular, intracellular pathogens for eliciting CTL specific for protective epitopes. A CTL response can be achieved through a number of strategies like immunization with synthetic peptides (Aichele et al., 1990), recombinant proteins (Kleid et al., 1981), vaccinia virus constructs (Mackett et al., 1985), soluble proteins osmotically vehicled to the cytosol (Carbone et al., 1990) and cells pulsed *in vitro* with synthetic peptides (external loading) (De Bruijn et al., 1991). While synthetic peptides are scarcely immunogenic and vaccinia vectors have drawbacks in previously vaccinated individuals (Lane et al., 1968), external loading of peptide provides a limited availability of empty class I MHC molecules at the cell surface.

The present research and invention is based upon the successful threshold experiment, producing model, novel immunoglobulin molecules found to be fully functional by virtue of their ability to localize on certain cell/receptor sites and elicit reactivity to the antigens specific for the introduced novel antigenic determinant or epitope. This invention demonstrates a new method for the engineering of cellular vaccines that can be used for the *in vivo* or *in vitro* induction of CTL.

A plasma cell can secrete about 10^3 molecules of Ig cell/sec, Ig can be an extraordinary source of endogenous peptides and B cells efficient APCs for presentation of peptide epitopes in the context of class I MHC molecules. In this invention, it is demonstrated that one can use B cells as APC to process and present a peptide from an endogenous Ig heavy (H) chain to a class I MHC restricted CTL clone specific for Ig peptide.

Summary of the Invention

The present invention is based upon the successful production of novel immunoglobulin molecules having introduced into the N-terminus variable region thereof a novel epitope not ordinarily found in the immunoglobulin molecule used as a starting molecule.

Successful model systems of the foreign molecules include the hydrophilic tetrapeptide Asn-Ala-Asn-Pro (NANP) of *Plasmodium falciparum* circumsporozite protein, the tripeptide Arg-Gly-Asp (RGD) involved in the interaction of a variety of adhesive proteins, and oligopeptide epitopes of the human CD4 HIV binding domain.

More particularly, this invention relates to introduction of oligopeptide epitopes of a nucleoprotein (NP) peptide of influenza virus for expression within the third fold of an immunoglobulin molecule. This virus peptide is recognized by CTL in the context of the H-2 D^b allele. It is shown that the NP peptide engineered in the H chain: 1) mediates killing of B cell lymphomas by a CTL clone specific for that peptide restricted by the D^b molecule, and 2) could be purified from the H-2 D^b molecules at the cell surface. This study formally demonstrates that peptides from the hypervariable loops of Ig are presented by class I MHC molecules and validates a role for the processing and presentation of self immunoglobulin V regions to $CD8^+$ T cells in the regulation of the immune response.

This invention demonstrates the possibility to program class I-restricted presentation of intracellular pathogens peptides using antigenized antibody genes as non-infectious "replicating" material .

The present invention is thus directed to novel immunoglobulin molecules having at least one novel heterologous epitope contained within the N-terminus variable domain thereof, said novel immunoglobulin molecule having retained functionality with respect to its C-terminus constant domain of the heavy chain specific for a particular cell/receptor type, and having novel, specific epitope _in vitro_ and _in vivo_ reactivity.

The present invention is further directed to pharmaceutical compositions containing, as essential pharmaceutical principal, a novel immunoglobulin hereof, particularly those in the form of an administrable pharmaceutical vaccine.

The present invention is further directed to methods useful for building tolerance to certain antigens, including those associated with autoimmune diseases, or for down-regulating hypersensitivity to allergens, or for providing active or passive immunity against certain pathogenic antigens, by administering to an individual in perceived need of such, a novel immunoglobulin molecule as defined above.

The present invention is further directed to novel recombinant means and methods useful for preparing, identifying and using the novel immunoglobulin molecules hereof including DNA isolates encoding them, vectors operatively harboring such DNA, hosts transfected with such vectors, cultures containing such growing hosts and the methods useful for preparing all of the above recombinant aspects.

Detailed Description of the Invention

The present invention is described herein with particular detail for the preparation of model, novel immunoglobulin entities. This description is provided, as it was conducted, using recombinant DNA technology. Further detail herein defines methods by which one can test a given immunoglobulin to assure that it exhibits requisite functionality common to its starting material immunoglobulin and specially as to its novel epitopic antigenic activity. Given this information with respect to the particular novel immunoglobulin molecules described herein, coupled with general procedures and techniques known in the art, the art skilled will well enough know how to configure recombinant expression vectors for the preparation of other novel immunoglobulin molecules falling within the general scope hereof for use as herein described. Thus, having described the threshold experiment of the successful preparation of a novel immunoglobulin molecule, one skilled in the art need not follow the exact details used for reproducing the invention. Instead, the art skilled may borrow from the extant, relevant art, known techniques for the preparation of still other novel immunoglobulin molecules falling within the general scope hereof.

1. Figure Legends

Figure 1 is a diagram illustrating the construction of the pNγ1NANP expression vector.

Figure 2 is an SDS-PAGE of the γ1NANP and WT recombinant Ig.

Figure 3 shows the binding of ^{125}I-labelled monoclonal antibody Sp3-B4 to engineered antibody γ1NANP.

Figure 4 is a Western blot binding of ^{125}-labelled antibody Sp3-B4 to engineered antibody γ1NANP and localization of the engineered $(NANP)_3$ epitope in the H chain.

Figure 5 shows results of cross-inhibition of ^{125}I-labelled antibody Sp3-84 binding to synthetic peptide $(NANP)_3$ (panel A) or engineered antibody γ1NANP (panel 8) by γ1NANP Ig or peptide $(NANP)_3$.

Figure 6 is a diagram of pNγ1NP expression vector and general strategy of transfection.

Figure 7 depicts specificity of target cell recognition by cold target competition. The inset shows the dose response of killing of B6-2 H^{NP} transfectants. ^{51}Cr-labeled B6-2.503 cells (2.5 x 10^5 cells/ml) were mixed with CTL clone 34 cells at an E:T ratio of 10:1, 1:1, 0.1:1 or 0:1. Percent cytotoxicity was calculated 4 hours later from triplicate cultures as described.

Figure 8 shows lack of interference of soluble γ1NP with external loading of peptide and lysis of target cells by a CTL clone.

Figure 9 shows inhibition of lysis of B6-2 H^{NP} transfectants by a monoclonal antibody to D^b.

Figure 10 shows presentation of processed NP peptide by engineered cells is restricted by H-2^b.

Figure 11 shows elution of influenza virus NP peptide from B6-2 H^{NP} tranfectants. (*a*) HPLC profile of the synthetic peptide ASNENMETM of influenza virus (100 μg). (*b*) Cytotoxic assay using single HPLC fractions from the experiment shown in *a*. (*c*) HPLC profile of the peptide mixture eluted from the D^b molecules purified from B6-2 HNP transfectants. (*d*) Cytotoxic assay using single HPLC

fractions from the experiment shown in *c*.

Figure 12 shows that addition of exogenous peptide does not increase lysis of cells engineered with the H^{NP} gene.

2. **General Methods and Definitions**

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, where such sequences are operatively linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors may be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. "Operative," or grammatical equivalents, means that the respective DNA sequences are operational, that is, work for their intended purposes. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified DNA sequence disposed therein is included in this term as it is applied to the specified sequence. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" referred to as circular double stranded DNA loops which, in their vector form, are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

Apart from the novelty of the present invention involving the introduction of novel epitopes by means of repositioning or augmentation of a parent immunoglobulin, it will be understood that the novel immunoglobulins of the present invention may otherwise permissively differ from the parent in respect of a difference in one or more amino acids from the parent entity, insofar as such differences

do not lead to a destruction in kind of the basic activity or bio-functionality of the novel entity.

"Recombinant host cells" refers to cells which have been transfected with vectors defined above.

Extrinsic support medium is used to support the host cells and includes those known or devised media that can support the cells in a growth phase or maintain them in a viable state such that they can perform their recombinantly harnessed function. See, for example, ATCC Media Handbook, Ed. Cote et al., American Type Culture Collection, Rockville, MD (1984). A growth supporting medium for mammalian cells, for example, preferably contains a serum supplement such as fetal calf serum or other supplementing component commonly used to facilitate cell growth and division such as hydrolysates of animal meat or milk, tissue or organ extracts, macerated clots or their extracts, and so forth. Other suitable medium components include, for example, transferrin, insulin and various metals.

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

"Heterologous" with reference herein to the novel epitope for a given immunoglobulin molecule refers to the presence of (at least one) such epitope in the N-terminus domain of an immunoglobulin that does not ordinarily bear that epitope(s) in its native state. Hence, that chain contains heterologous epitope sequence(s). Such heterologous epitope sequences shall include the classic antigenic epitopes as well as receptor like binding domains or binding regions that function as receptor sites, such as the human CD4 binding domain for HIV, hormonal receptor binding ligands, retinoid receptor binding ligands and

ligands or receptors that mediate cell adhesion.

"Chimeric" refers to immunoglobulins hereof, bearing the heterologous epitope (s), that otherwise may be composed of parts taken from immunoglobulins of more than one species. Hence, a chimeric starting immunoglobulin hereof may have a hybrid heavy chain made up of parts taken from corresponding human and non-human immunoglobulins.

In addition to the above discussion and the various references to existing literature teachings, reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques encompassed by the present invention. See, for example, Maniatis, et al, <u>Molecular Cloning: A Laboratory Manual</u>, Cold Spring Harbor Laboratory, New York, 1982 and the various references cited therein, and in particular, Colowick et al., <u>Methods in Enzymology</u> *Vol. 152*, Academic Press, Inc. (1987). All of the herein cited publications are by this reference hereby expressly incorporated herein.

The foregoing description and following experimental details set forth the methodology employed initially by the present researchers in identifying and characterizing and preparing particular immunoglobulins. The art skilled will recognize that by supplying the present information including the wherewithal of the location and makeup of the epitope containing domain of a given immunoglobulin, and how it can be manipulated to produce the novel immunoglobulins hereof. Therefore, it may not be necessary to repeat these details in all respects in their endeavors to reproduce this work. Instead, they may choose to employ alternative, reliable and known methods, for example, they may synthesize the underlying DNA sequences encoding a particular novel immunoglobulin hereof for deployment within similar or other suitable, operative expression

vectors and culture systems. Thus, in addition to supplying details actually employed, the present disclosure serves to enable reproduction of the specific immunoglobulins disclosed and others, and fragments thereof, such as the individual chains for in vitro assembly, using means within the skill of the art having benefit of the present disclosure. All of such means are included within the enablement and scope of the present invention.

3. Description of Particularly Preferred Embodiments

Protein engineering was used to introduce a foreign epitope into the CDR3 of the H chain of a mouse/human chimeric antibody ($C\gamma_1 62$). This epitope consists of three copies of the tetrapeptide Asn-Ala-Asn-Pro (NANP). The tetrapeptide occurs naturally as a 37 tandem repeat in the Plasmodium falciparum circumsporozoite (CS) protein, interspersed with four repeats of the variant sequence Asn-Val-Asp-Pro [Dame et al., Science *229*:593 (1984)]. In the construct described here, the epitope is flanked by Val and Pro residues at each end [VP $(NANP)_3$ VP]. The experiment verified that the $(NANP)_3$ epitope could be inserted in the NV region of a host H chain (V_H) without altering the framework folding of the Ig molecule, i.e., its molecular assembly with the light (L) chain and it determined that the antigenic and immunogenic properties of the recombinant Ig molecule were expressed. It is known that the CDR3 of V^H regions of antibody is often the structural correlate of an immunodominant idiotope [Davie, et al., Ann. Rev. Immunol. 4:147 (1986)], which indicates that the CDR3 is at the surface of the molecule. Moreover, it is well established that because of recombination of the variable-diversity-joining (VDJ) regions, as well as N-addition mechanisms [Tonegawa, Nature *302*:575 (1983); Miller et al., Immunol. Today 7:36 (1986)], the CDR3 may vary considerably in length (from 3 to 19 amino acids) [Kabat, et al.,

Proteins of Immunological Interest, U.S. Dept. of Health and Human Service NIH (1987)], implying a high degree of plasticity at the structural level. Second, the $(NANP)_3$ epitope selected for this study is relatively short, repetitive and of proven immunogenicity in mice and humans [Good et al., Ann. Rev. Immunol. 6:663 (1988)].

Particularly developed is a new method for the presentation of immunogenic epitopes to cytotoxic T lymphocytes (CTL). The new method is based on antibody antigenization, a process whereby one or several loops of an immunoglobulin molecule are re-engineered to encompass the sequence of selected portion of pathogens (virus and parasites), self antigens and tumor antigens.

Antibodies may be antigenized by inserting immunogenic epitopes in any of the three CDR regions of each heavy chain and any of the three CDR regions of the light chain. A preferred site of engineering an immunogenic epitope is the third CDR region of the heavy chain.

An immunogenic epitope may be inserted into one or more of the six CDRs, thus generating an antibody antigenized with between one and six epitopes. In a preferred embodiment, one immunogenic oligopeptide sequence is engineered within the third complementarity-determining region (CDR3) of the heavy chain of the immunoglobulin.

Immunogenic epitopes may be engineered within any or all of the CDRs by inserting a nucleic acid sequence encoding the epitope at a site unique to the CDR and absent from the nucleic acid sequence of the immunoglobulin chain wherein the epitope sequence is to be inserted. Insertion may be accomplished, for example, using a restriction enzyme capable of recognizing the unique sequence in the CDR.

Mature lymphocytes of the CD8 phenotype recognize antigen in conjunction with class I MHC molecules. The best studied systems relate to CTL that recognize virally-infected cells (Long et al., Immunol. Today 10:45, 1989). CTL's function requires active replication of the virion within the cell. Few examples do however, exist to indicate that inactivated (non-replicating) virus (Wraith et al., J. Gen. Virol. 66:1327, 1985) or soluble proteins (Moore et al., Cell 54:777 (1988); Staerz et al., Nature 329:449 (1987)) can also be presented to class I-restricted T cells provided that they reach the inside of the cell.

To further demonstrate the invention, an antibody was engineered to encompass the oligopeptide sequence ASNENMETMESSTL representing a CTL epitope of influenza virus nucleoprotein (NP) (Bastin et al., J. Exp. Med. 165:1508, 1987; Townsend et al., Cell 44:959 (1986)). This epitope has been characterized as a short nonglycosylated protein sequence which is recognized as a target by NP-specific CTL clones in a MHC-restricted way. More recently, it has been directly proven that this peptide is indeed responsible for targeting CTL on influenza virus-infected cells as it could be eluted from the MHC class I molecule (Rotzschke et al., Nature 348:2 (1990)).

4. **Examples**

Example I

A. Construction of the pNγ1NANP expression vector

The production of hybridoma 62 and B10H2, and the purification of mAb 62 and 109.3 (anti-2,4-dinitrophenol) have been described previously [Zanetti et al., J. Immunol. *131*:2452 (1983) and Glotz et al., J. Immunol. *137*:223 (1986)].

A DNA library was constructed from size-selected 2-2.5-kb Eco RI fragments from hybridoma 62 genomic DNA. Fragments were eluted from low melting point agarose and ligated into

the λgt10 vector [Huynh et al., DNA Cloning Techniques *1*:49 (1985)]. After ligation and packaging, 5 x 10^4 plaque-forming units were screened by replicate hybridization with the J_H [Sakano et al., Nature *286*:676 (1980)] and pSAPC15 [Brodeur et al., Eur. J. Immunol. *14*:922 (1984)] probes. Four clones were isolated and plaque purified; the 2.3-kb EcoRI insert form one of them was subcloned into pEMBL18 vector [Dente et al., DNA Cloning Techniques *1*:101 (1985)]. The V_HB10H2 coding sequence was determined by cloning the cDNA from the parental hybridoma by primer extension of the poly(A)$^+$ RNA with a synthetic oligonucleotide (5'-GGGGCCAGTGGATAGAC-3') that anneals at the 5' end of the CH1 region. The same oligonucleotide was used as a probe for screening the library after 5' end-labeling by kinase with ^{32}P-ATP. The nucleotide sequence of both clones was determined by dideoxy method on both strands after subcloning suitable restriction fragments into the pEMBL18 vector.

Plasmid pN$\gamma_1$62 containing DNA encoding C_1, 62 antibody was constructed by subcloning in the proper orientation the 2.3-kb EcoRI DNA fragment carrying the V_H62 rearrangement into the unique EcoRI site of the PNγ_1 vector [Sollazo et al., Focus *10*:64 (1988)] (a PSV derived vector harboring an human γ^1, gene). This vector encodes a human γ_1 gene downstream from the EcoRI site. It also carries a neomycin resistance gene under the control of the SV40 promoter for the selection of stable transformant cells. Transfectoma cells were constructed by introducing the plasmids pN$\gamma_1$62 and pNγ_1CHA, a chimeric construct encoding an antibody lacking Id62 and Ig binding into J558L mouse by electroporation. This cell line is an H chain-defective variant of myeloma J558 [Morrison et al., Science *229*:229 (1985)] and carries the rearrangement for a λ1 light (L) chain. Briefly 3 x 10^6 cells in 1 ml of Dulbecco's modified minimum essential medium (DMEM) containing 10 μg of supercoiled plasmid DNA were pulsed for 17 ms at 650

V/cm in a Cell Porator apparatus (Bethesda Research Laboratories, Bethesda, MD). After pulsing, the cells were resuspended in 10 ml of DMEM supplemented with 10 mM Hepes buffer, 2 mM L-glutamine, penicillin (50 μg/ml), streptomycin (50 μg/ml) and 10% fetal calf serum (cDMEM), and incubated for 48 h at 37°C in a 10% CO_2 atmosphere. The cells were then resuspended in 20 ml of cDMEM and an aliquot (2 ml) was diluted into 20 ml of cDMEM containing 1.2 mg/ml of G418 (Gibco, Grand Island, NY), plated on a 96-well microtiter plate and cultured for 14 days. The supernatants of neomycin-resistant colonies (stable transformants) were tested by solid-phase radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA).

The presence of Id62 in the supernatant of J558L cells transfected with p$N\gamma_1$62 vector was tested by competitive inhibition in ELISA. This measures the inhibition (percent) of the binding of horseradish peroxidase (HP)-conjugated mAb 62 (ligand) to anti-Id62 antibody coated on 96-well polyvinyl microtiter plate (Dynatech, Alexandria, VA) [Zanetti et al., J. Immunol. *131*:2452 (1983)]. The supernatant of J558L cells transfected with p$N\gamma_1$CHA plasmid and purified mAb 62 and 109.3 (an IgG_1, x anti-2,4-dinitrophenol) served as controls [Zanetti et al., J. Immunol. *131*:2452 (1983)]. A second method to test for Id62 expression was by Western blot [Towbin et al., Proc. Natl. Acad. Sci. USA *71*:4350 (1979)]. Briefly, approximately 5 μg of antibody c$\gamma_1$62 purified by affinity chromatography on an anti-human Ig Sepharose 4B column (Pharmacia, Uppsala, Sweden) was electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. The gel was then blotted onto 0.45 M nitrocellulose paper (Millipore, Bedford, MA) and probed with ^{125}I-labelled affinity-purified syngeneic anti-Id62 antibody [Zanetti et al. J. Immunol. *135*:1245 (1985)]. Antibodies 62 and 109.3 served as

positive and negative control, respectively. The filter was exposed a first time for 24 h at -70°C with intensifier screen. To demonstrate the co-expression of the human C region on the H chain of the chimeric $c\gamma_1 62$ antibody, the nitrocellulose paper was re-probed with ^{125}I-labelled goat anti-human Ig antibody and exposed for 2 h at 70°C.

Sequence data is publicly available from EMBL/Gene Bank Data Library under Accession No. Y00744.

The γ1NANP antibody carrying the malarial CS immunodominant B-cell epitope NANP in the CDR3 of its H chain was engineered as follows:

Figure 1 is a diagram illustrating the construction of the pNγ1NANP expression vector. In panel A: (a) The productively rearranged V_H gene of the hybridoma cell line 62 isolated from a size-selected lambda gt10 library and subcloned into pBluescript (publicly available from Stratagene, San Diego, CA) is described infra.; (b) The restriction site KpnI/Asp718 of the polylinker region was deleted by Kpn I digestion, filled in with T4 polymerase and ligated, yielding the plasmid pH62Δk; (c) pH62Δk was used as a template for site-directed mutagenesis to introduce a unique Asp718 restriction site in CDR3 of the V_H gene. The synthetic oligonucleotide (5-'CAAGAAAGGTACCCTACTCTC-3'), which encodes a 3 bp insertion (TAC), was annealed to the uracylated single-stranded complementary template and elongated; (d) Complementary synthetic oligonucleotides

(5'-GTACCCAATGCAAACCCAAATGCAAACCCAAATGCAAACCCA-3'
3'- GGTTACGTTTGGGTTTACGTTTGGGTTTACGTTTGGGTCATG-5')

were annealed and subcloned into the unique Asp718 site of pH62k. The construction was verified by sequence analysis by using a 15^{mer} primer corresponding to the 5' end of V_H62 gene (5'-GACGTGAAGCTGGTG-3'); (e) The 2.3-

kb Eco RI fragment carrying the engineered V_HNANP gene was subcloned upstream from the human γ1 C region into the 15-kb pNγ1 vector. The pNγ1NANP construct was electroporated into J558L cells subsequently cultured in the presence of G418. Resistant clones were screened for Ig production by a sandwich enzyme-linked immunosorbent assay (ELISA) using goat anti-human antibodies immobilized on microtiter wells as the capturing antibodies and horseradish peroxidase (HP) conjugated goat anti-human Ig (Sigma) as the revealing antibodies. Clones producing >2-5 μg Ig/ml of protein 10^6 cells were expanded and the antibody purified from culture supernatants. Sequence modifications illustrated in panel A are shown in detail in panel B. Abbreviations used: Asp - Asp 718; B - BamHI; RI - EcoRI; FR - framework region; CDR - complementarily-determining region; neo - neomycin (G418) resistance; amp - ampicillin resistance.

The restriction fragment encoding the V_H gene of a murine monoclonal antibody to thyroglobulin (mAb 62) was modified as shown in Figure 1. A double-stranded synthetic DNA fragment encoding three copies of the NANP tetramer $(NANP)_3$ and carrying Asp718 protruding ends was inserted in frame between Pro 95 and Tyr 96 of V_H62k coding region. The pH62NANP construct was verified by dideoxy sequencing. The Eco RI restriction fragment encoding the engineered Vg was subcloned into the pNY_1 expression vector upstream from the human y1 constant (C) region to obtain the $pN\Gamma_1$NANP construct. This plasmid was electroporated into the murine J558L cell line, a H chain-defective variant of myeloma J558L that carries the rearrangement for a lambda-1 L chain [Morrison et al., <u>Science</u> *229*:1202 (1985)].

Transfectoma cells were cultured, subcloned and screened for secretion of the engineered Ig molecule

using a sandwich enzyme-linked immunosorbent assay (ELISA) with goat anti-human Ig antibodies. Clones producing 2-5 μg/ml of protein 10^6 cells were selected and expanded, and the chimeric protein was purified by means of affinity chromatography on a Sepharose 48-Protein-A column. The purified Ig molecule was analyzed by SDS-PAGE under reducing and nonreducing conditions.

Figure 2 is an SDS-PAGE of the γ1NANP and WT recombinant Ig. Five μg of Protein A-purified antibody were loaded on a 7.5% polyacrylamide gel under nonreducing conditions. The gel was stained with Comassie blue. The inset shows the resolution into heavy (H) and light (L) chains of engineered antibody γ1NANP electrophoresed on a 10% polyacrylamide gel under reducing (5% β-mercaptoethanol) conditions.

Figure 2 shows that the nonreduced γ1NANP chimeric antibody has an apparent molecular weight of 160 kD, suggesting a proper H_2L_2 assembly to form a tetrameric Ig protein. When the γ1NANP antibody was compared with the wild-type (WT) Ig, a chimeric antibody lacking the $(NANP)_3$ insert, purified from culture supernatant fluid of J558L cells transfected with $pN\gamma_{162}$, a slight difference in size was observed due to the presence of the inserted epitope. However, the molecular weight of the γ1NANP antibody is well in the range of a tetrameric complex. Both preparations also showed a smear in the region below the 160 kD band, suggesting some degradation and/or noncorrectly assembled protein products. Under reducing conditions, the engineered γ1NANP antibody was appropriately resolved into an H and na L chain (Figure 2, inset). As determined by ELISA of NP-40 lysates, transfectoma cells secreting the γ_1NANP antibody had approximately the same cytoplasmic levels of H chains as cells producing the

WT Ig. Collectively, these results indicate that the insertion of 15 amino acids into the CDR3 of V_H62 did not appreciably alter the interaction between V_H and V_L polypeptide chains nor the assembly and secretion of the tetrameric (H_2L_2) Ig molecule.

B. <u>Binding of monoclonal antibody Sp-3-B4 to engineered antibody γ1NANP</u>

To determine if the engineered γ1NANP antibody indeed expresses the $(NANP)_3$ epitope in an immunological accessible form, solid-phase radioimmunoassay (RIA) and Western blot techniques were used and a murine monoclonal antibody (Sp3-B4) generated against <u>P. falciparum</u> and specific the NANP epitope.

Figure 3 shows the binding of ^{125}I-labelled monoclonal antibody Sp-3-B4 to engineered antibody γ1NANP. Murine monoclonal antibody (mAb) Sp3-B4, an IgG2a,k antibody produced by immunization with the <u>P. falciparum</u> parasite and reacting with the repetitive epitope NANP. Specific for the NANP epitope, any antimalainal antibody could be so used as a tool and generated via analogous techniques. Polyvinyl microtiter wells were coated by drying at 37°C with 5 μg/ml solution in 0.9% NaCI of purified γ1NANP Ig (solid diamonds), WT (solid triangles), $(NANP)_3$ synthetic peptide (solid squares), a 16^{mer} synthetic peptide (YYCARKAYSHGMDYW) encompassing the CDR3 of the V_H region of prototype antibody 62 (open squares), and the 15^{mer} synthetic peptide YPQVTRGDVFTMPED of the cell-adhesive molecule vitronectin (open diamonds). The ^{125}I-labelled antibody Sp3-B4 (20 x 10^4 cpm/50μl) was incubated overnight at +4°C. After extensive washing, the bound radioactivity was counted in a gamma counter. The test was done in triplicate.

The results of the direct RIA binding (Figure 3) showed that ^{125}I-labelled mAb Sp3-B4 bound both the synthetic peptide $(NANP)_3$ and the recombinant γ1NANP antibody immobilized on microtiter wells. However, the binding to antibody γ1NANP can be considered more efficient; in molar terms, the estimated ratio of peptide to antibody was about 50 to 1, assuming that the antibody expresses two copies of the $(NANP)_3$ epitope per Ig molecule. No binding occurred to either the WT Ig or two irrelevant synthetic peptides, one corresponding to the CDR3 sequence of prototype V_H62 and the other to residues YPQVTRGDVFTMPED of vitronectin.

Figure 4 is a Western blot binding of ^{25}I-labelled antibody Sp3-B4 to engineered $(NANP)_3$ epitope in the H chain. Ten μg of purified γ1NANP Ig, recombinant WT Ig, native monoclonal antibody 62, and polyclonal human gamma globulins (HGG) (Cohn fraction II, Miles) were loaded onto a 10% SDS-PAGE and electrophoresed at 150 V under nonreducing (left panel) and reducing (right panel) conditions. Resolved proteins or polypeptide chains were transferred from the gel to 0.45-μm nitrocellulose paper. After blotting, the filter was blocked with 10% solution of dry milk in 0.9% NaCI for two hours at room temperature. The sheet was then incubated overnight at +4°C by rocking with ^{125}I-labelled antibody Sp3-B4 (40×10^4 cpm/ml) in phosphate-buffered saline, pH 7.3, containing 1% bovine serum albumin and 1% Tween 20. After incubation, the filter was washed extensively, dried and exposed to Kodak XAR-5 film at -70oC for 18 hours. Binding to γ1NANP Ig, recombinant WT Ig, antibody 62 and HGG in RIA by the same $_{125}$I-labelled probe (10^5 cpm/50μl) was 10,560; 420; 360; and 330 cpm, respectively.

Western blot analysis (Figure 4) showed that ^{125}I-labelled mAb Sp3-B4 specifically bound antibody γ1NANP

in both the nonreduced (left panel) and reduced (right panel) forms. In the latter, as expected, binding occurred on the H- but not the L-chain, confirming that the engineered γ1NANP antibody bears the $(NANP)_3$ epitope on the H chain. No binding occurred to controls for the H and L chain and the human C region.

C. <u>Efficiency of engineered γ1NANP antibody in expressing the $(NANP)_3$ epitope</u>

A cross-inhibition assay was employed to assess the engineered γ1NANP antibody's relative efficiency in expressing the $(NANP)_3$ epitope. The synthetic peptide $(NANP)_3$ and antibody γ1NANP were used to inhibit the binding of ^{125}I-labelled mAb Sp3-B4 to either the $(NANP)_3$ peptide or the γ1NANP antibody immobilized on microtiter plates.

Figure 5 shows results of cross-inhibition of ^{125}I-labelled antibody Sp3-B4 binding to synthetic peptide $(NANP)_3$(panel A) or engineered antibody γ1NANP (panel 8) by γ1NANP Ig or peptide (NANP) A fixed amount of ^{125}I-labelled antibody Sp3-B4 (probe) was mixed vol/vol with decreasing amounts of the various inhibitors diluted in phosphate-buffered saline, pH 7.3, containing 1% bovine serum albumin and 1% Tween 20. The mixture was incubated at +4°C overnight by rocking. Fifty μl of each mixture were incubated on individual polyvinyl microtiter wells coated with either synthetic peptide $(NANP)_3$ (panel A) or purified engineered γ1NANP Ig (panel B). The conditions of coating are as detailed in the legend to Figure 4. The following inhibitors were used: purified γ1NANP Ig, WT Ig, and synthetic peptides $(NANP)_3$, CDR3 and vitronectin. The percentage of inhibition was calculated as follows: [(average binding of the probe alone) - (average binding of the probe incubated in the presence of

inhibitor)]/(average binding of the probe alone) x 100. Tests were done in duplicate.

Figure 5 shows that both the peptide and the engineered antibody efficiently inhibited the binding to both physical forms of the $(NANP)_3$ epitope, i.e., synthetic peptide and antibody borne. However, whereas the γ1NANP antibody was about four times more effective than the peptide itself (panel A) in inhibiting binding to the synthetic peptide, it was approximately 150 times more effective than the peptide in inhibiting binding to the engineered Ig (panel B). The WT Ig and control peptides (CDR3 and vitronectin) caused no inhibition. Thus, when compared with the synthetic peptide it appears that the $(NANP)_3$ epitope borne on the γ1NANP antibody assumes a three-dimensional configuration that in immunological terms more closely mimics that of the active CS protein.

D. Induction *in vivo* of anti-NANP antibodies by recombinant γ1NANP antibody

To determine whether the recombinant γ1NANP antibody could be used to induce anti-NANP antibodies, in vivo experiments were performed in rabbits. Two rabbits were immunized with the engineered γ1NANP antibody, and two controls receive the WT Ig. As indicated in Table I, infra., as early as 30 days after the first immunization, both rabbits immunized with the γ1NANP antibody produced anti-NANP antibodies detectable by ELISA and RIA. After booster immunizations, the titer rose in both rabbits; the maximal titer was 1/3200 on day 70. Importantly, this antiserum was positive when tested by indirect immunofluorescence on P. falciparum sporozoite showing that the epitope expressed by the γ1NANP Ig is indeed mimicking the native antigen. Sera from control rabbits immunized with the WT Ig did not

react with the $(NANP)_3$ peptide immobilized on microtiter wells nor with the parasite. Rabbits of both groups produced an anti-human response as determined by agglutination of red cells coated with human gamma globulin. Rabbits' antisera were tested by direct immunofluorescence on P. falciparum (strain Indochina III) dried onto glass slides in the presence of 10% fetal bovine serum.

The observation that the V_H region of an antibody molecule can be engineered to express 15 amino acid residues containing an epitope of an unrelated molecule shows that the V_H/C_H polypeptide chain containing the foreign epitope is properly assembled with the endogenous L chain to form a (H_2L_2) tetramer, so it appears that the insertion of this epitope in the CDR3 was tolerated and did not affect the overall Ig framework folding. Based upon the present research, as long as the recombinant epitope is stereochemically compatible with contiguous CDR residues, it can be inserted or substituted for a CDR and can be expected to be exposed at the surface of the molecule, although it cannot be ruled out that the results reported here may be due to the nature of the epitope itself. In the construct described here, the $(NANP)_3$ sequence is flanked on both sides by the amino acids Val and Pro. Possibly, this helps stabilize the inserted epitope by anchoring it at each end. The large ramification at the $C\beta$ atom and the $C\gamma$-methyl group of the Val residue may hinder the main chain by decreasing its flexibility; the side chain of Pro by curling back to the main chain seizes it, leading to the formation of an almost rigid side chain.

Studies in vitro using the binding site of a NANP-specific monoclonal antibody as a probe for the protein-surface interaction and in vivo demonstrating

that rabbits immunized with the engineered Ig molecule produce anti-NANP antibodies that react with the plasmodium antigen show that the $(NANP)_3$ epitope expressed by the engineered Ig is both antigenic and immunogenic. In other terms, neither the molecular environment nor the globular folding of Ig modified the immunologic structure of the $(NANP)_3$ epitope. From a biological standpoint, the $(NANP)_3$ epitope engineered into an Ig molecule can be viewed as an idiotope a la carte built into the CDR3 of a host V_H domain. Based on what is known of the immunogenicity of idiotypes and the predictable events that follow induction of immunity via the idiotype network [Jerne Ann. Immunol. (Paris) *125*:373 (1974); Cozenave et al., PNAS *74*:5122 (1977); Urbain et al., PNAS *74*:5126 (1977); Bona et al., J. Exp. Med *153*:951 (1981)], these results imply that an immune response of predetermined epitope specificity can be dictated in molecular terms and predicted in vitro. This strategy can be exploited to render a B-cell epitope T-independent, proving its utility not only for analyses of the structure and function of epitopes and Igs but also for the development of new antibody vaccines, for example, as an alternative to peptide based vaccines. Preparation of vaccines may be accomplished using extant methodology, already developed for immunoglobulins as such.

TABLE I

Induction of Anti-NANP Antibodies in Rabbits Immunized with the Engineered γ1NANP Antibody[a]

Rabbit No.	Immunogen	Days After Immunization				
		0	30*	40	60*	70
44	WT	0	ND	0	0	0
45	WT	0	ND	0	0	0
49	γ1NANP	0	1/100	1/400	1/400	1/3200
50	γ1NANP	0	0	1/400	1/200	1/1600

[a]Adult white rabbits were immunized subcutaneously in several points of the back with 50 μg of recombinant γ1NANP or the WT antibody emulsified in complete Freund's adjuvant (CFA). Booster injections of 50 μg of the same immunogen in incomplete Freund's adjuvant were given at monthly intervals (denoted by an asterisk). Sera were collected on the days indicated and tested for reactivity with the synthetic $(NANP)_3$ peptide by solid-phase ELISA and RIA. Briefly, serial two-fold dilutions of individual sera in phosphate-buffered saline, pH 7.3, containing 1% bovine serum albumin and 1% Tween 230 were incubated overnight at +4°C on microtiter plates coated with the $(NANP)_3$ peptide at 5 μg/ml in 0.9% NaCI. After the incubation, the plates were washed and incubated with either a horseradish peroxidase conjugated goat anti-rabbit Ig, or ^{125}I-labelled Protein A (Amersham) for one hour at room temperature. Next, the plates were washed and the bound antibodies determined by using a Bio-Rad (Richmond, CA) ELISA reader or a gamma counter. The binding of the preimmune sera was considered the reference background value. The titer was determined from the mean binding of triplicate samples after subtracting the background binding values and is expressed as the reciprocal serum dilution.

Example II

Materials and Methods

Monoclonal antibodies

The murine monoclonal antibody 28-14-8S (γ2a, k) specific for H-2^b (D^b allele) was purchased from the American Tissue Type Collection (ATCC No. HB27). Fluorescein-conjugate murine monoclonal antibody AMS-32.1 reacting with I-A^d was purchased from Pharmigen (San Diego, CA).

Synthetic Peptides

A/PR/8/34 influenza virus nucleoprotein synthetic peptide ASNENMETM (amino acid residues 366-374) was synthesized on an ABI 430-A automated synthesizer (Applied Biosystems, Inc., Foster City, CA).

Cells

B6-2 is a nonsecreting murine B cell hybridoma (H-$2^{d,b}$) originally established by fusing C57Bl/6 (B6) (H-2^b) splenic B cell with M12.4.1 lymphoma cells of BALB/c (H-2^d) origin, and were kindly obtained from Dr. R. Abe (National Institute of Health, Bethesda, MD). J558L is a murine myeloma of BALB/c (H-2^d) origin and is a H-chain defective variant of J558 myeloma carrying the rearrangement for a λ_1 light (L)-chain (Morrison, Science 229:1202 (1985)). J558L cells lack constitutive Ig secretion, but they secrete a H_2L_2 Ig molecule when transfected with a H-chain gene. The non-secreting Sp2/0 myeloma (H-2^d) was obtained through passage from ATCC No. CRL 1581. $CD8^+$ murine CTL clone 34 (Vitiello et al., J. Immunol. 143:1512 (1989); Vitiello et al., J. Immunol. 131:1635 (1983)) is specific for the monopeptide ASNENMETM (residues 366-374) of the nucleoprotein (NP) antigen of A/PR8 influenza virus (Bastin et al., J. Exp. Med. 165:1508 (1987); Rotzschke et al., Nature 348:252 (1990);

Townsend et al., Cell 44:959 (1986)), and is restricted by the class I histocompatibility D^b gene product. The clone was maintained in culture by stimulation at weekly intervals with irradiated syngeneic spleen cells pulsed with the ASNENMETM synthetic peptide.

Engineering techniques

The D region of the parental V_H gene (KAYSHG; residues 93-98) was mutagenized (Sollazzo et al., Eur. J. Immunol. 19:453 (1989)) to introduce a single KpnI/Asp718 site to yield the intermediate sequence KVPYSHG (residues 93-99). The amino acid 94A was deleted and substituted by the VP doublet encoded by the nucleotide sequence of the Asp718 cloning site. Subsequently, complementary oligonucleotides 5' GTA CCC GCT TCC AAT GAA AAT ATG GAG ACT ATG GAA TCA AGT ACA CTT 3', 5' GTA CAA GTG TAC TTG ATT CCA TAG TCT CCA TAT TTT CAT TGG AAG CGG 3' coding for residues 366-379 of the influenza nucleoprotein (NP) (ASNENMETMESSTL) were introduced between 94V and 95P of the mutagenized V_H region. The engineered V_HNP coded by the 2.3 kb EcoRI fragments was cloned upstream from a human γ1 constant (C) region gene contained in the 12.8 kb vector pNγ1 (Sollazzo et al., Eur. J. Immunol. 19:453 (1989)). Thirty μg of the DNA construct pNγ1NP were electroporated in B6-2, J558L and Sp2/0 cells (2×10^7) using a field strength of 625 V/cm. Transfected cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, 0.1 mM non-essential amino-acids, 1 mM sodium pyruvate, 0.1 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.5 μM β-mercaptoehanol for 24 hours, and then selected in the presence of neomycin (0.8 mg/ml) (G418; Gibco-BRL).

Esterase Release Assay

B6-2 H^{NP} transfectants cells were screened for presentation of the NP peptide by using an immunoenzymatic method that measures the release of esterase by CTL upon specific peptide antigen recognition (Kane et al., Mol. Immunol. 26:759 (1989); Pasternack et al., Nature 322:740 (1986)). Briefly, 10^5 effector cells (CTL clone 34) and 10^4 cells from each transfectoma were coincubated in a final volume of 100 μl of culture medium in 96-well flat-bottom plates. Untransfected B6-2 cells and B6-2 cells pulsed with the NP peptide (5 μg/ml) served as negative and positive controls, respectively. Spontaneous and maximum esterase release were assessed on effector cells incubated in medium alone or in the presence of 1% Triton 100-X. After 4 hours of incubation at 37) °C, the plates were centrifuged of 400 rpm for 2 min and the supernatants collected. Twenty five microliters of each supernatant were transferred to 96-well flat-bottom plates to which were added 175 μl of phosphate buffered saline (PBS) containing 2 x 10^{-4} M N-benzyloxycarbonyl-L-lysine thiobenzil ester and 2.2 x 10^{-4} M 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma, St Louis, MO). After an incubation period of 30 min. at 25 °C the absorbance at 412 nm was determined in an ELISA plate reader. The results were expressed in percent secretion as follows: [sample secretion - spontaneous secretion / maximum secretion - spontaneous secretion] x 100.

Cytotoxicity Assay

Cytotoxicity was tested in a 4 hours ^{51}Cr release assay. Briefly, target cells were labeled with $Na^{51}CrO_4$ (150 μCi/1 x 10^6 cells) for 1 hour at 37 °C in an atmosphere of 5% of CO_2 with or without NP peptide (10 μg/ml or as specified), then washed and resuspended in culture medium supplemented with 10% FCS. One hundred ml of

^{51}Cr-labeled target cells (2.5×10^5 cells/ ml) were mixed with 100 μl of CTL clone 34 (effector cells) at an effector:target cells (E:T) ration of 10:1, or as specified. The plates were incubated for 4 hours at 37 °C in 5% CO_2, then centrifuged at 500 *g* for 4 minutes. One hundred microliters of supernatant was removed and counted in a gamma counter. Spontaneous and maximal ^{51}Cr release were determined by incubating target cells in medium alone or in the presence of 1% Triton X-100, respectively. The cytotoxic activity was calculated from triplicated wells as follows: [experimental release - spontaneous release / maximal release - spontaneous release] x 100. Cold target competition was done by mixing 50 μl of ^{51}Cr-labeled B6-2.503 cells (5×10^5 cells/ml) with 50 μl of EI-4 or B6-2 cells pulsed with NP peptide (10 μg/ml) at a cold:hot cell ratio of 0:1, 5:1, 25:1, and 50:1. Then, 100 μl of CTL clone 34 (effector cells) were added at a E:T ratio of 10:1. Percent cytotoxicity was calculated 4 hours later as described above. γ1NP, or γ1NANP as negative control, was added at a final concentration of 100 μg/ml either during the pulsing or the cytotoxicity phase. The murine monoclonal antibody 28-14-8S (γ2a,κ) specific for $D^{b,q}$ or a mouse monoclonal antibody of the same isotype but of unrelated specificity as control was added during the cytotoxic assay at a final concentration of 50, 5, and 0.5 μg/ml, respectively. HPLC fractions were tested for their capacity to pulse B6-2 cells as follows: 100 μl of ^{51}Cr-labeled B6-2 cells (2.5×10^5 cells/ml) were mixed with 5 μl of each fraction. After 1 hour incubation at 37 °C, 100 μl of CTL clone 34 (effector cells) were added at a E:T ratio of 10:1. The NP peptide (10 μg/ml) was used as positive control.

Isolation of NP peptide from the class I histocompatibility D^b surface molecule

The NP peptide was isolated from B6-2 H^{NP} transfectants by acid elution. Briefly, D^b-specific monoclonal antibody 28-14-8S (γ2a,k) was immobilized on Protein-A beads at a ratio of 500 µl of beads:3 mg of 28-14-8S antibody for 1 hour at 4 °C. Bulk cultures of 10% stable B6-2 H^{NP} transfectants (clone 514 or 503) were pelleted and resuspended at 2 x 10^8 cells/ml in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 5mM EDTA) containing a freshly-made cocktail of proteases inhibitors [A-Protinin (5 µg/ml), Leupeptin (10 µg/ml), Pepstatin-A (10 µg/ml), and PMSF 1 mM] for 30 min. at 4 °C. Nuclei were pelleted by centrifugation at 3,000 *g* for 15 min. Lysates were then mixed with antibody 28-14-8 S/Protein-A immunosorbent by rocking for 1 hour at 4 °C. Protein-A beads conjugated with the influenza peptide/D^b complexes were then pelleted by centrifugation at 1,000 *g* for 5 min., washed three times with wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP40, 5 mM EDTA) followed by a wash with PBS and HPLC-grade H_2O. The NP peptides were acid-extracted by washing the Protein A-class I MHC molecules beads twice with 0.2% trifluoroacetic acid (TFA). The low molecular weight material was separated by filtration through a Centricon 10 titer (Amicon) with a molecular weight cut off of 10,000 kD. The filtrate was lyophilized and kept at -20 °C until used.

HPLC analysis of peptides

Low molecular weight material containing peptides purified from D^{b+} B6-2-H^{NP} transfectants (clones 514 cr 503) were analyzed by reverse phase HPLC using a SMART System unit (Pharmacia) and a mRPC C2/C18 2.1 /10 column (Pharmacia). Peptides were eluted using 0.1% TFA in H_2O (v/v) (solution A) and 0.08% TFA in

acetonitrile (solution B). The flow rate was 100 μl/min and fractions of 100 μl were collected. The following gradient conditions were used: 0-61 min. a linear increase to 60% B; 61-66 min. 60% B; 66-71 min. increase to 100% B; 71-76 min. decrease to 0% B. One hundred micrograms of synthetic NP peptide (residues 366-374) was purified using the same conditions and served as a reference.

A. <u>Generation of B cells that present the influenza virus NP peptide (residues 366-379) to a CTL clone specific for the same viral peptide</u>

A H chain gene was engineered to encompass in the third complementarity-determining region (CDR3) a nucleotide sequence encoding for the amino acid sequence ASNENMETMESSTL (residues 366-379) of influenza virus NP antigen through the process of antibody antigenization (Zanetti, <u>Nature</u> 355:466 (1992)). The engineered H chain gene (H^{NP}) was used to transfect B6-2 (H-$2^{d,b}$), SP2-0 (H-2^{d}) and J558L (H-2^{d}) cells, respectively. J558L cells carry the gene for the λ1 light chain and served to produce a H_2L_2 antibody molecule.

The H chain plasmid is the product of the fusion of a human γ1C region with a murine V_H engineered to express the NP sequence 366-379 in CDR3. The coding strand of the CDR3 region is shown in bold, with the NP-coding sequence underlined. The amino acid sequence of the influenza peptide ^{366}ASNENMETMESSTL^{379} is shown in bold. B, BamHI; RI, EcoRI; Neo, neomycin (G418) resistance; Amp, ampicillin resistance. The DNA construct (pNγ1NP) was electroporated in the murine B6-2 (H-$2^{d,b}$) B cell hybridoma to generate target cells. The stable transfectants were initially screened using a serine-esterase release assay to select clones that could activate CTL clone 34 to release serine esterase used as a cellular probe (peptide presentation). The selected clones were then tested in a conventional ^{51}Cr-

release assay; clones that confirmed positive were expanded. Alternatively, the DNA construct was electroporated into murine myeloma cell line J558L (H-2^d), a H chain-defective variant of myeloma J558 carrying the rearrangement for a λ1 L chain. Supernatants of neomycin-resistant colonies (stable tranfectants) were tested by ELISA for Ig production. The final product is a H_2L_2 molecule γ1NP (antigenized antibody).

Neomycin-resistant B6-2 hybrid transfectants were screened for their ability to present the NP peptide to CTL clone 34 specific for the 9^{mer} ASNENMETM sequence. To simultaneously screen a large number of transfectants, a serine esterase-release assay (Pasternack et al., Nature 322:740 (1986)) was used. This assay is a readily-detectable indicator of secretory granules exocytosis triggered in the CTL clone by specific recognition of the NP peptide/class I MHC molecule complex on B6-2 H^{NP} transfectants. Five out of 120 (4%) transfectants induced a release of esterase equivalent to that of B6-2 cells pulsed with a synthetic NP peptide (Table 2). B6-2 cells transfected with a wild type H chain gene (H^{WT}) -lacking the NP epitope- and B6-2 cells alone served as negative controls. To confirm the results of the esterase-release assay a conventional ^{51}Cr-release assay was used. This assay showed an absolute correlation between the two tests (Table 2). In order to select transfectants with stable integration of the H^{NP} gene primary transfectants were subcloned and retested using the same strategy. It is worth noting that transfectants negative by both esterase- and ^{51}Cr-release assays remained negative throughout subsequent tests. On the other hand, positive transfectants maintained their ability to present the NP epitope during a twelve month period.

TABLE II

Selection of B6-2 H^{NP} tranfectants that process and present the NP peptide

			B6-2 clones				
Cell Line	B6-2	B6-2NP	D3	2.503	2.504	2.514	2.522
Gene Transfected	None	None	H^{WT}	H^{NP}	H^{NP}	H^{NP}	H^{NP}
Serine Esterase-Release *	11%	49%	N.D.	44%	34%	35%	40%
^{51}Cr Release **	2%	60%	8%	63%	38%	52%	40%

* The serine esterase-release assay was done as detailed in the Material and Methods.

** Percent cytotoxicity was determined in 4-hours ^{51}Cr release assay at an effector:target cells ratio of 10:1.B6-2^{NP} cells were pulsed for 1 hour at 37°C with NP peptide (10μg/ml). ND:not done. For all cells maximum release was at least 10 times the minimum release.

B. Specificity of the killing of engineered cells by the CTL clone

To ascertain the specificity of lysis of B6-2 H^{NP} transfectants by the CTL clone cold target competition experiments were performed using B6-2 and EL-4 cells pulsed *in vitro* with the synthetic NP peptide (10 μg/ml) as the cold inhibitor.

^{51}Cr-labeled B6-2.503 cells (5 x 10^5 cells/ml) were mixed with EI-4 or B6-2 cells pulsed or not with NP peptide (10 μg/ml) at a cold:hot target cell ratio of 0:1, 5:1, 25:1 and 50:1, respectively. Then, CTL (effector cells) clone 34 were added at a E:T ratio of 10:1. Percent cytotoxicity was calculated 4 hours later from triplicate wells as described. Maximum and minimum ^{51}Cr release were 48, 197±1,177 and 6,137±93 cpm, respectively.

As shown in Figure 7 in both instances there was complete inhibition of cytolysis at a competitor: target ratio of 50:1. This demonstrates that killing of B6-2 lymphoma cells engineered with the H^{NP} gene was specific for the NP peptide/class I MHC molecule complex.

C. Soluble antigenized antibody expressing the NP epitope in CDR3 does not interfere with presentation of processed peptide from the endogenous H^{NP} chain

Although B6-2 is a nonsecreting B cell lymphoma, it is not unprecedented that transfection with a H chain gene reactivates a latent L chain gene. For instance, threshold amounts of antibody that may be undetectable in our assay (< 1 ng/ml) could have been endocytosed, processed and presented. To rule out this possibility, it was verified whether or not soluble antigenized antibody could mediate lysis of B6-2 cells.

^{51}Cr-labeled EI-4 cells (5×10^5 cells/ml) were mixed with NP peptide and/or γ1NP (or γ1NANP as control) at a final concentration of 10 or 100 μg/ml, respectively. Then, CTL clone 34 (effector cells) was added at an E:T ratio of 10:1. Intact γ1NP molecule (or γ1NANP as control) was added during the cytotoxic phase of the assay at a final concentration of 100 μg/ml. Percent cytotoxicity was calculated 4 hours later from triplicate wells as described.

As shown in Figure 8 untransfected B6-2 cells pulsed with γ1NP were not lysed. Moreover, the antigenized antibody added to H^{NP} transfectants during pulsing with peptide did not affect lysis nor did it modify the percent of lysis when added during the lytic phase.

These results demonstrate that lysis of B6-2 H^{NP} transfectants is the result of presentation of a processed peptide derived from the endogenously synthesized H^{NP} chain. It should be pointed out that lack of direct influence on CTL lysis suggests that the whole antibody does not function as an anti-receptor antibody.

D. <u>Lysis of B6-2 H^{NP} transfectants is restricted by the D^b allele</u>

The role of the D^b allele in the presentation of the NP peptide to the CTL clone by the engineered B cells wa analyzed using a twofold approach. First, it was ascertained that a murine monoclonal antibody (28.14.8S) specific for the D^b allele could block cytotoxicity.

^{51}Cr-labeled B6-2.503 cells (2.5×10^5 cells/ml) were mixed with CTL clone 34 (effector cells) at an E:T ratio of 10:1 in the presence of various doses of the murine monoclonal antibody 28.14 (κ,γ2a) specific for

$D^{b,q}$. A mouse monoclonal antibody of the same isotype, but of unrelated specificity, was used as control. Percent cytotoxicity was calculated 4 hours later from triplicate wells as described. Figure 9 shows a dose-dependent inhibition by antibody 28.14.8S, but not by an isotype-matched control antibody.

Second, a series of H^{NP} transfectants carrying the H-2^d haplotype (Sp2/0 and J558L cells) were analyzed. ^{51}Cr-labeled B6-2 (H-$2^{b,d}$), Sp2/0 (H-2^d) or J557L (H-2^d) cells pulsed with NP peptide (10 μg/ml) or transfected with H^{NP} or H^{WT} (2.5 x 10^5 cells/ml), were mixed with CTL clone 34 (effector cells) at an E:T ratio of 10:1. Percent cytotoxicity was calculated 4 hours later from triplicate wells as described. As illustrated in Figure 10 lysis of these cells did not exceed that of control H^{WT} transfectants. Thus, the NP peptide resulting from the proteolytic fragmentation of the endogenous H^{NP} chain is presented in association with D^b allele as if it were generated from an intracellular replicating virus.

E. <u>Purification of viral NP peptide from B6-2 cells transfected with the H^{NP}</u> gene

It was important to demonstrate that the NP peptide could be purified from surface class I D^b molecules and that it could be used to pulse untransfected B6-2 cells and mediate lysis by CTL clone 34. A lysate of 10^9 B6-2 H^{NP} transfectants was mixed with Protein-A Sepharose beads coated with monoclonal antibody 28.14.8S. MHC-bound peptides were extracted by acid elution using 0.2% TFA, and the peptides were separated from class I MHC molecules by centrifugation on a low molecular weight Centricon filter. The low molecular weight material was fractionated by reverse-phase HPLC. The elution profile of a representative experiment is shown

in Figure 11 (A and C). The HPLC profile of the control 9^{mer} synthetic peptide ASNENMETM is shown as a comparison. Individual fractions were used to pulse untransfected ^{51}Cr-labelled B6-2 cells to identify the fraction(s) containing the NP peptide. Figure 11 (panel B and D) shows that in both instances the active peptide was eluted in fractions 19 and 20, suggesting that the peptide purified from the B6-2 H^{NP} transfectants has physicochemical and biological characteristics similar to the 9^{mer} synthetic peptide ASNENMETM. Thus, a proteolytic fragment of the endogenously-synthesized H^{NP} chain bound the D^b allele, was transported at the cell surface and mediated lysis by the NP-specific CTL clone.

F. <u>Quantitative analysis of processing and presentation of the NP peptide in B6-2 cells engineered with the H^{NP}</u>

That B6-2 and H^{NP} transfectants were killed efficiently by a specific CTL clone and the NP peptide could be eluted from the cell surface of these cells prompted a quantitative analysis of this phenomenon. First, the effect of exogenous addition of synthetic peptide NP on the lysis of B6-2 H^{NP} transfectants was verified.

^{51}Cr-labeled B6-2 or B6-2.503 cells were pulsed with 0.1, 1 or 10 μg of NP peptide for 1 hour at 37 °C, then mixed with CTL clone 34 (effector cells) at an E:T ratio of 10:1. Percent cytotoxicity was calculated 4 hours later from triplicate wells. As shown in Figure 12 excess amounts (10 $\mu g/ml$) of NP peptide added at the beginning of the cytotoxicity assay failed to induce an increase of lysis, hence implying that occupancy of MHC class I molecules by processed peptide from the endogenous H^{NP} chain was already maximum.

The foregoing description details specific methods that can be employed to practice the present invention.

Having detailed specific methods initially used to identify, isolate, characterize, prepare and use the immunoglobulins hereof, and a further disclosure as to specific model entities, the art skilled will well enough know how to devise alternative reliable methods for arriving at the same information and for extending this information to other intraspecies and interspecies related immunoglobulins.

For example, antigen sequences can be engineered at any restriction site unique to the CDR sequence within which the antigen sequence is to be inserted, and absent from the sequence of the immunoglobulin chain wherein the CDR is located. Unique sequences in the six CDRs can be identified and located using a combination of known immunoglobulin nucleic acid sequences and cleavage sites of restriction enzymes. Further, a desired unique restriction site may be introduced into the CDR wherein the antigenic determinant is to be inserted using molecular techniques well known to those skilled in the art. In addition, it is well within the knowledge of those skilled in the art to modify the present invention by, for example, engineering an antigen within any of the six complementarity-determining regions of an immunoglobulin.

Thus, however detailed the foregoing may appear in text, it should not be construed as limiting the overall scope hereof; rather, the ambit of the present invention is to be governed only by the lawful construction of the appended claims.

Claims:

1. An immunoglobulin molecule containing at least one nucleoprotein (NP) peptide of influenza virus within a complementarity-determining region (CDR), said immunoglobulin molecule having the effector function conferred by the constant region of the immunoglobulin, and having specific influenza virus NP epitope reactivity.

2. An immunoglobulin molecule according to Claim 1, wherein said CDR is the third CDR in the N-terminus variable domain of said molecule.

3. An immunoglobulin according to Claim 1 wherein said nucleoprotein peptide comprises residues 366 to 374 of the influenza nucleoprotein.

4. As a product of recombinant DNA technology, an immunoglobulin according to Claim 1.

5. A heavy chain of an immunoglobulin containing within the third complementarity-determining region (CDR) in the N-terminus variable domain thereof at least one influenza virus nucleoprotein peptide.

6. A pharmaceutical composition comprising as an essential principle an immunoglobulin molecule according to Claim 1.

7. The composition according to Claim 6 suitable for administration to a human subject.

8. The composition according to Claim 6 in the form of an administrable vaccine.

9. A DNA molecule that is a recombinant DNA molecule

or a cDNA molecule encoding an immunoglobulin molecule according to Claim 1.

10. An expression vector operatively harboring DNA encoding an immunoglobulin, defined according to Claim 9.

11. A recombinant host cell transfected with an expression vector according to Claim 10.

12. A process of preparing an immunoglobulin molecule according to Claim 1 which comprises expressing in a recombinant host cell transfecting DNA encoding said immunoglobulin molecule.

FIG. 1A

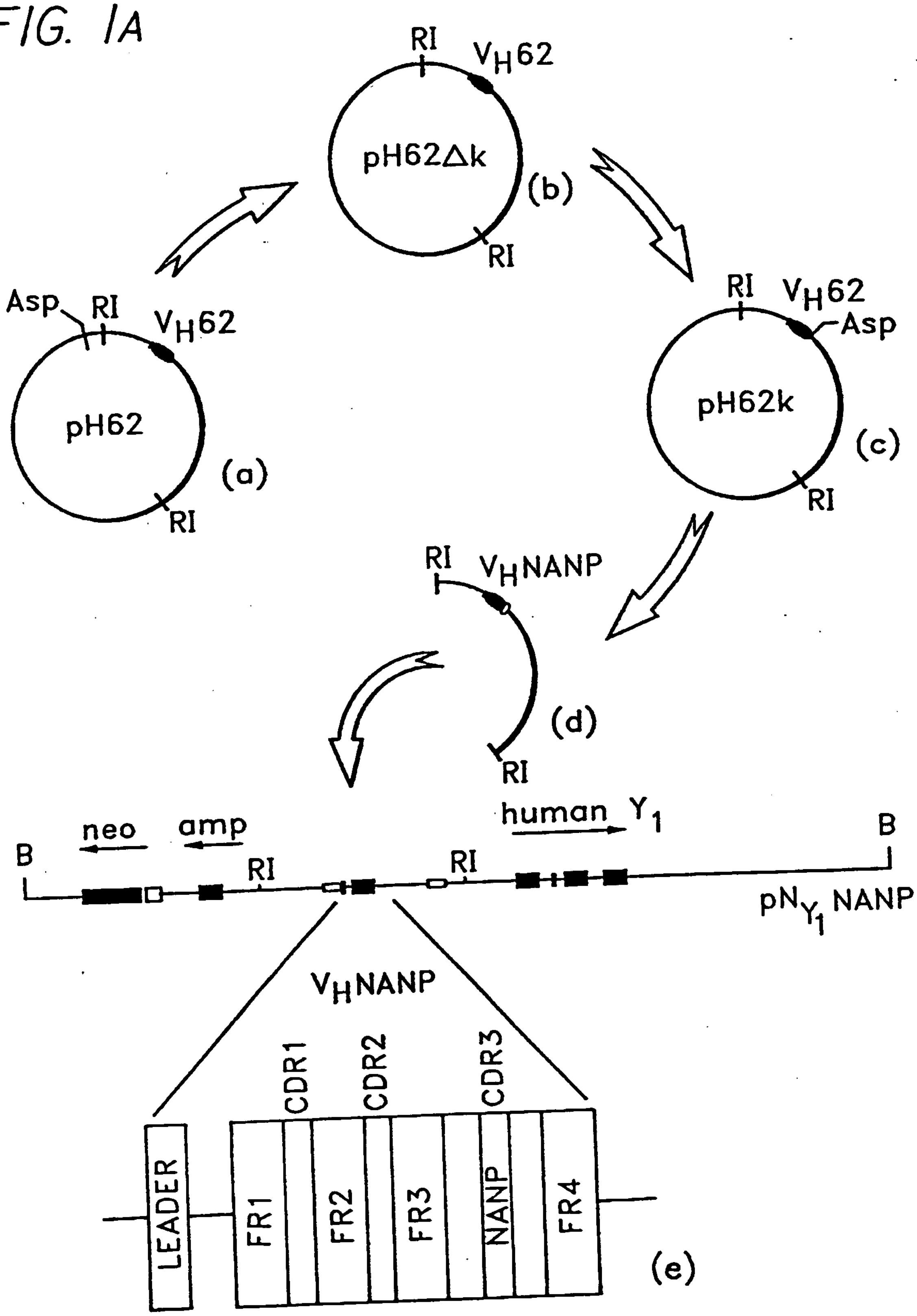

RI
V_H62
pH62Δk
(b)
RI
Asp
RI
V_H62
pH62
(a)
RI
RI
V_H62
Asp
pH62k
(c)
RI
RI
V_HNANP
(d)
RI
neo
amp
human γ_1
B
RI
RI
B
$pN_{\gamma_1}NANP$
V_HNANP
CDR1
CDR2
CDR3
LEADER
FR1
FR2
FR3
NANP
FR4
(e)

FIG. 1B

pH62Δk

```
90                          |------------- CDR3 -------------|
 C   A   R   K   A   Y   S   H   G   M   D   Y   W
TGT GCA AGA AAG GCC TAC TCT CAT GGT ATG GAC TAC TGG
```

pH62k

```
90                          |----------------- CDR3 -----------------|
 C   A   R   K   V   P   Y   S   H   G   M   D   Y   W
TGT GCA AGA AAG GTA CCC TAC TCT CAT GGT ATG GAC TAC TGG
                ---------
                 Asp718
```

pH NANP

```
90                          |------------------------------- CDR3 -------------------------------|
 C   A   R   K   V   P   N   A   N   P    V   P   Y   S   H   G   M   D   Y   W
TGT GCA AGA AAG GTA CCC [AAT GCA AAC CCA]3 GTA CCC TAC TCT CAT GGT ATG GAC TAC TGG
```

FIG. 2

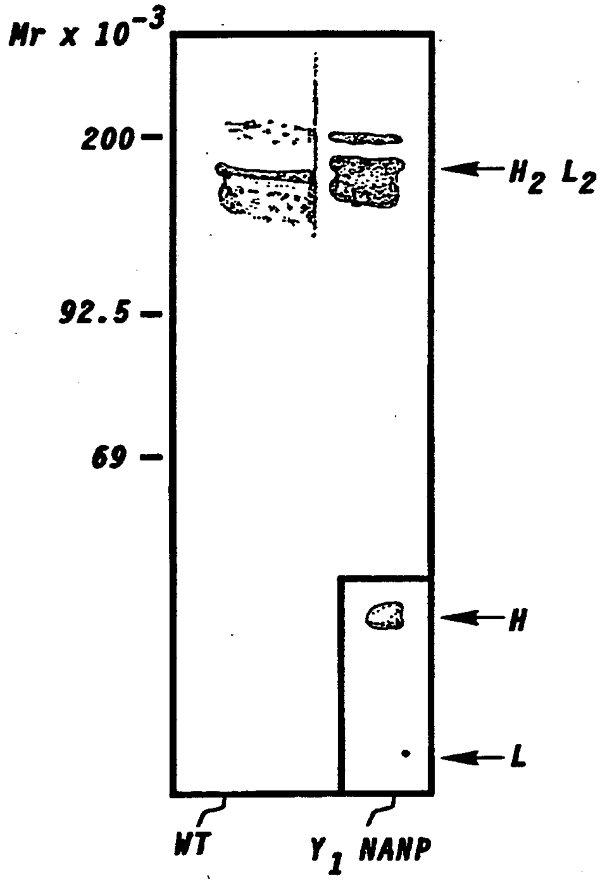


FIG. 3

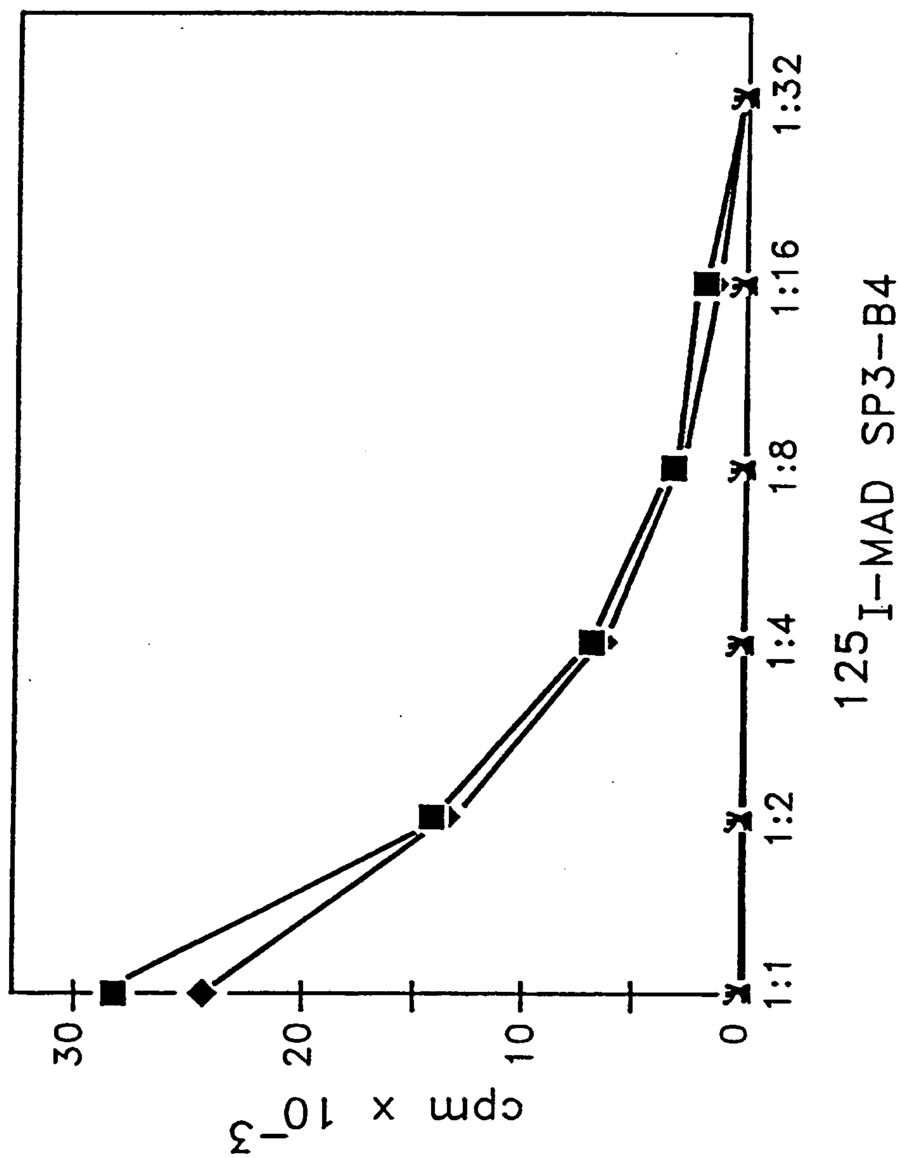


FIG. 4A

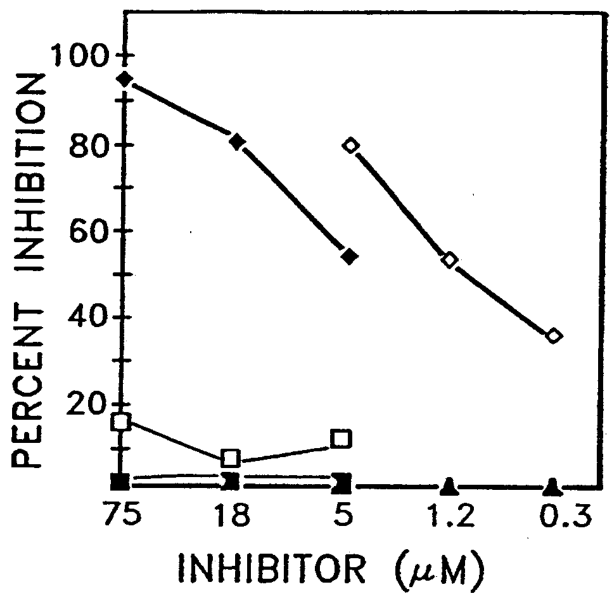
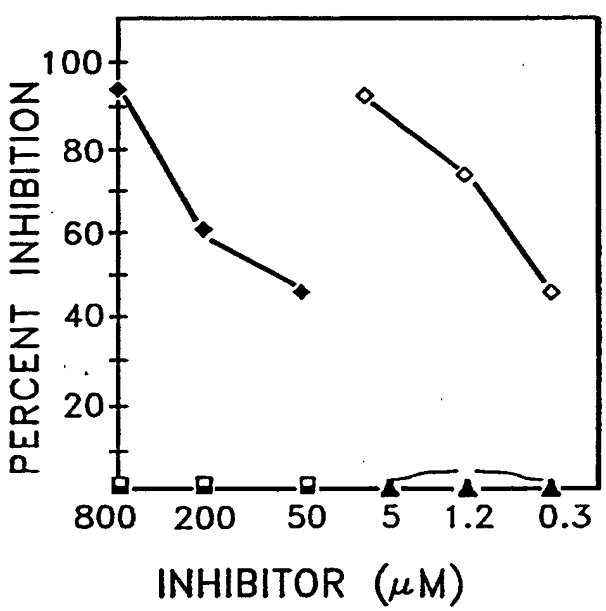

PERCENT INHIBITION
100
80
60
40
20
75
18
5
1.2
0.3
INHIBITOR (μM)

FIG. 4B


PERCENT INHIBITION
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80
60
40
20
800
200
50
5
1.2
0.3
INHIBITOR (μM)

FIG. 5

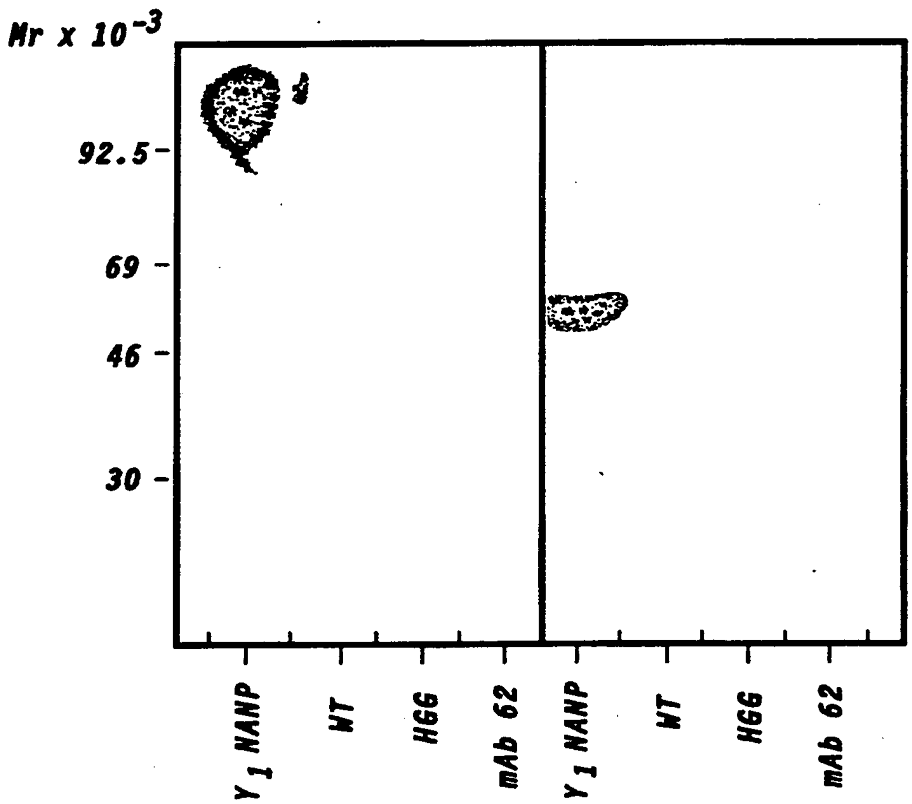


FIG. 6

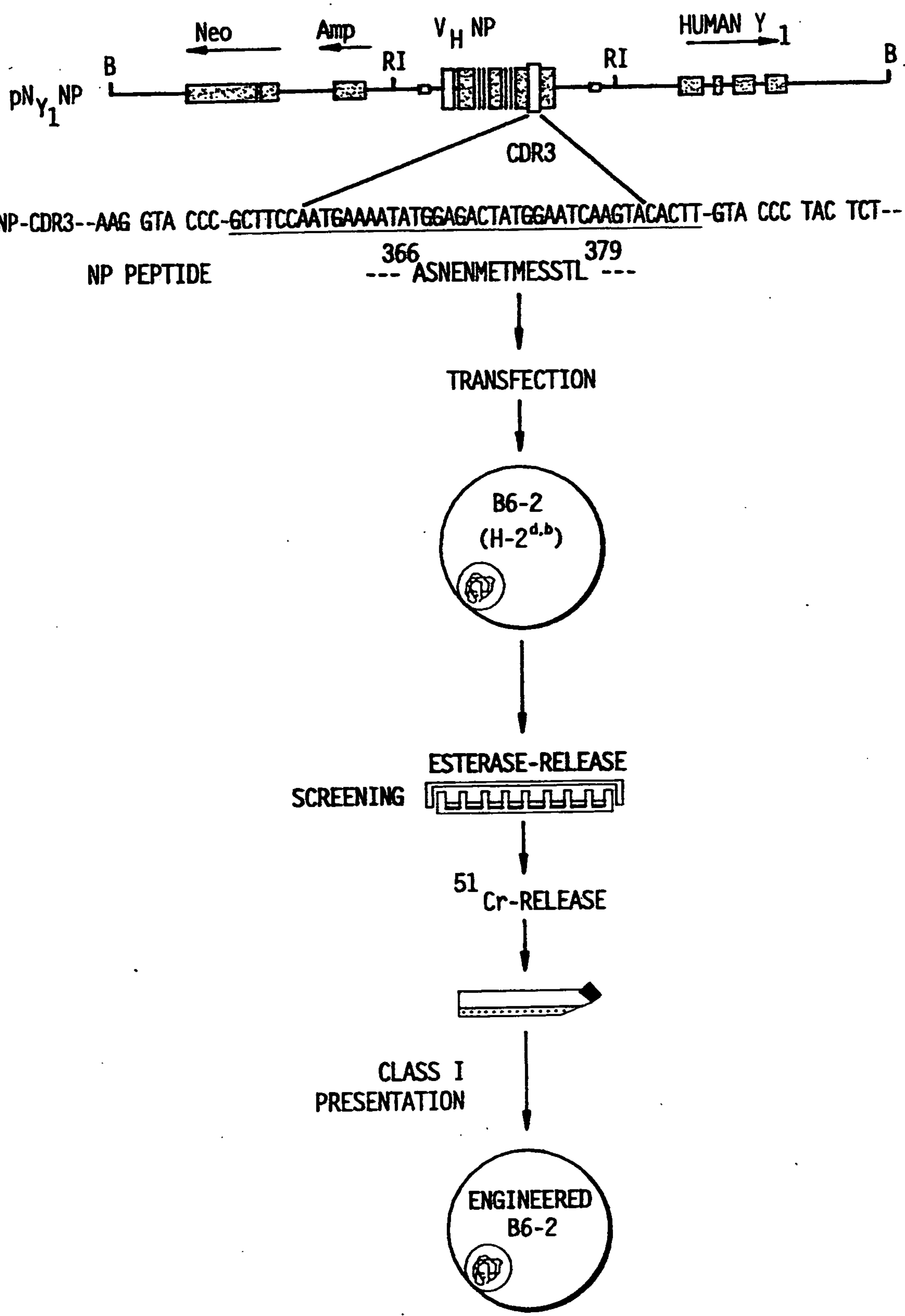
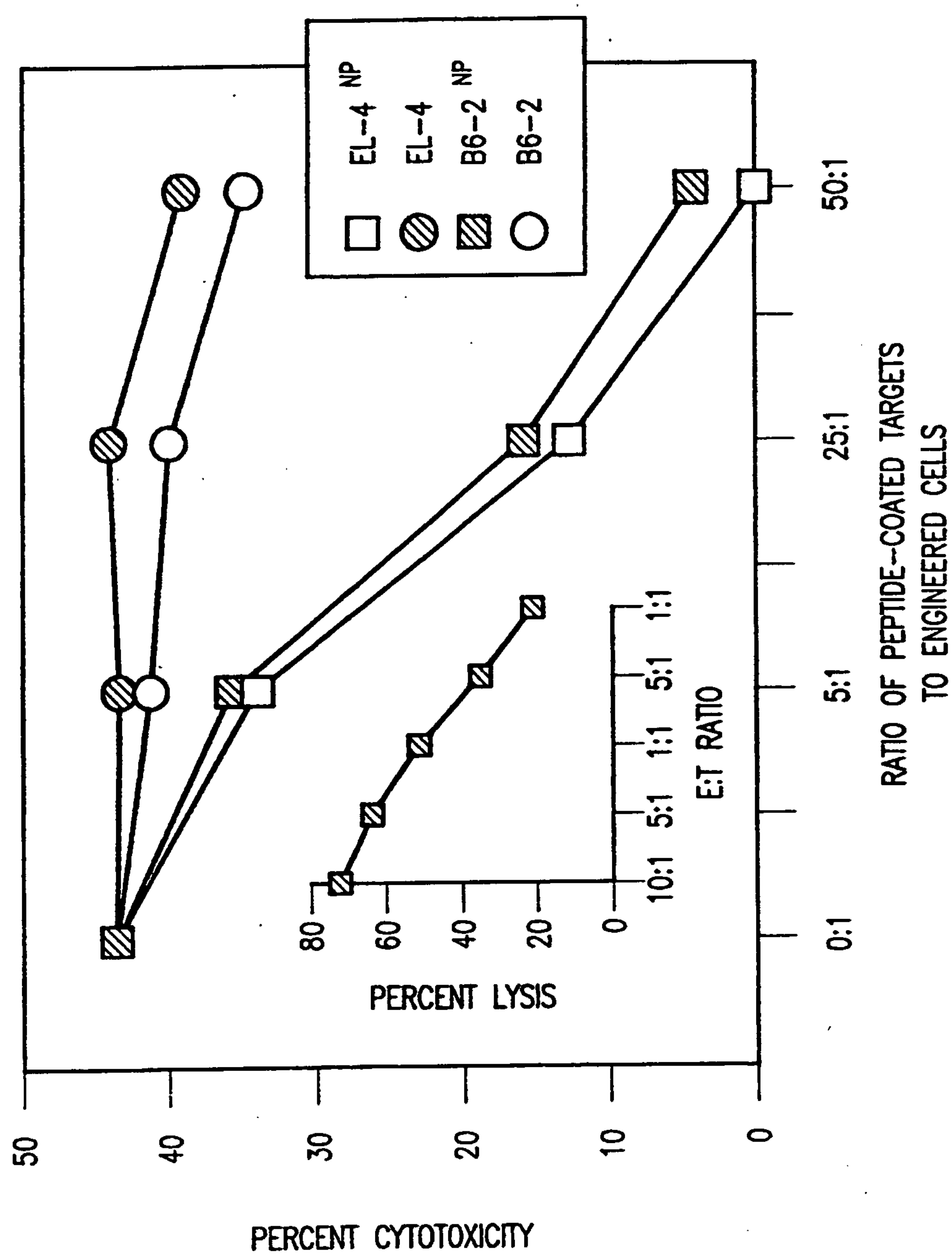

Neo
Amp
V_H NP
HUMAN γ_1
B
RI
RI
B
$pN\gamma_1NP$
CDR3
VHNP-CDR3--AAG GTA CCC-GCTTCCAATGAAAATATGGAGACTATGGAATCAAGTACACTT-GTA CCC TAC TCT--
366
379
NP PEPTIDE
--- ASNENMETMESSTL ---
TRANSFECTION
B6-2
(H-2^{d,b})
ESTERASE-RELEASE
SCREENING
51
Cr-RELEASE
CLASS I
PRESENTATION
ENGINEERED
B6-2

FIG. 7


EL-4 NP
EL-4
B6-2 NP
B6-2
PERCENT CYTOTOXICITY
50
40
30
20
10
0
0:1
5:1
25:1
50:1
RATIO OF PEPTIDE-COATED TARGETS TO ENGINEERED CELLS
PERCENT LYSIS
80
60
40
20
0
10:1
5:1
1:1
5:1
1:1
E:T RATIO

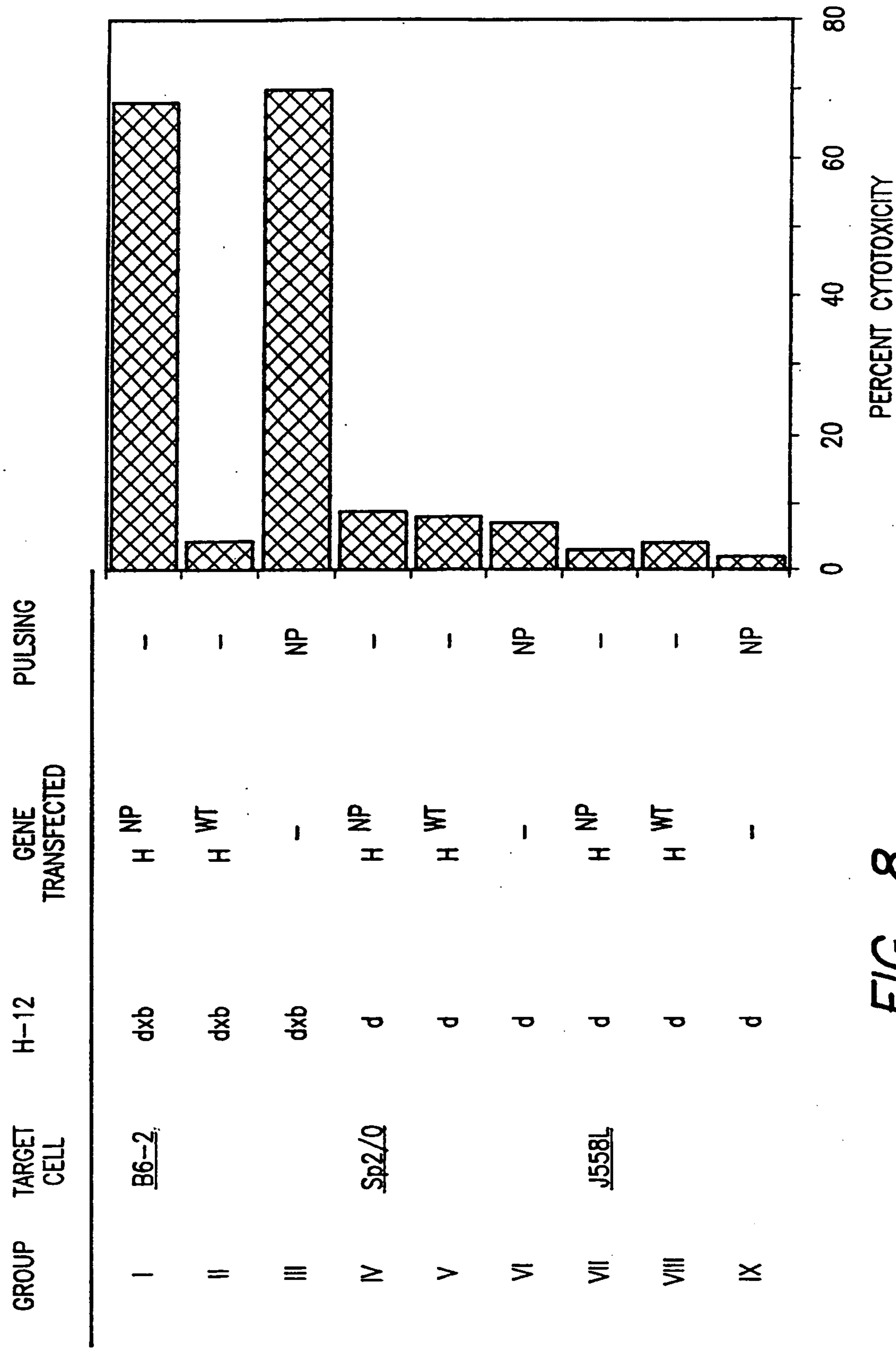
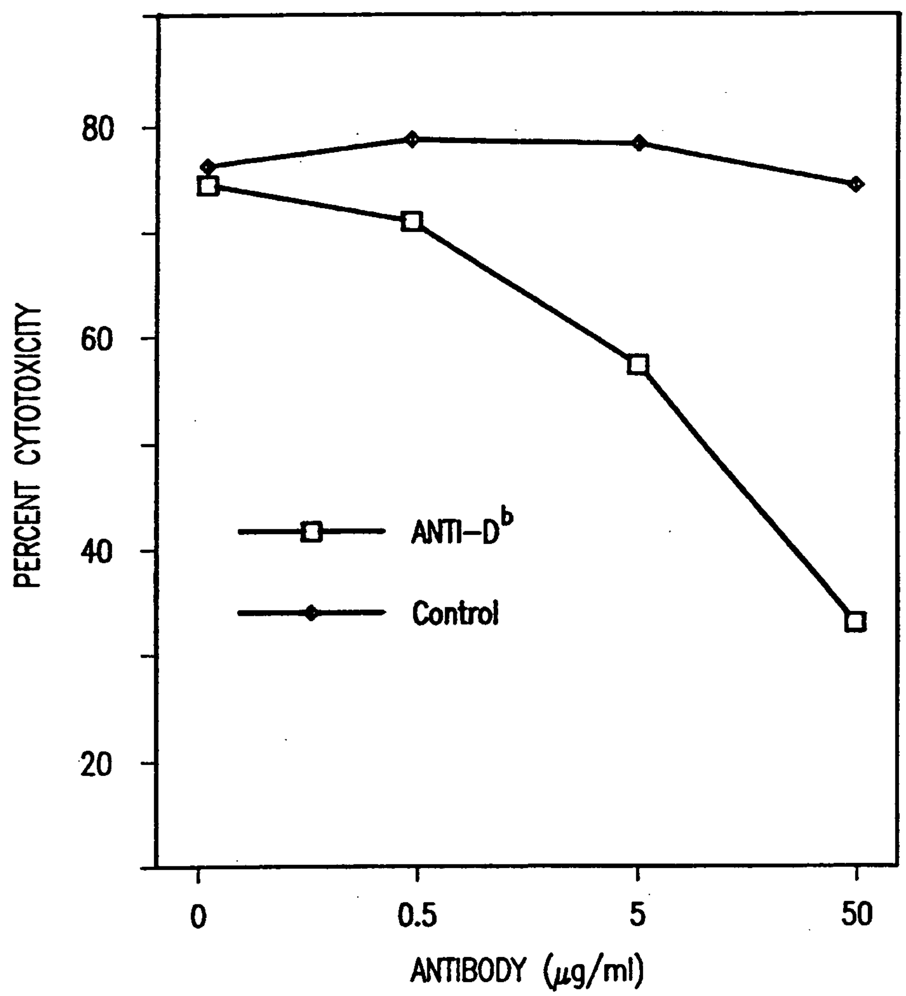

GROUP
TARGET CELL
H-12
GENE TRANSFECTED
PULSING
I
II
III
IV
V
VI
VII
VIII
IX
B6-2
Sp2/0
J558L
dxb
d
H NP
H WT
NP
PERCENT CYTOTOXICITY
0
20
40
60
80

FIG. 8

FIG. 9


80
60
40
20
PERCENT CYTOTOXICITY
ANTI-D^b
Control
0
0.5
5
50
ANTIBODY (μg/ml)

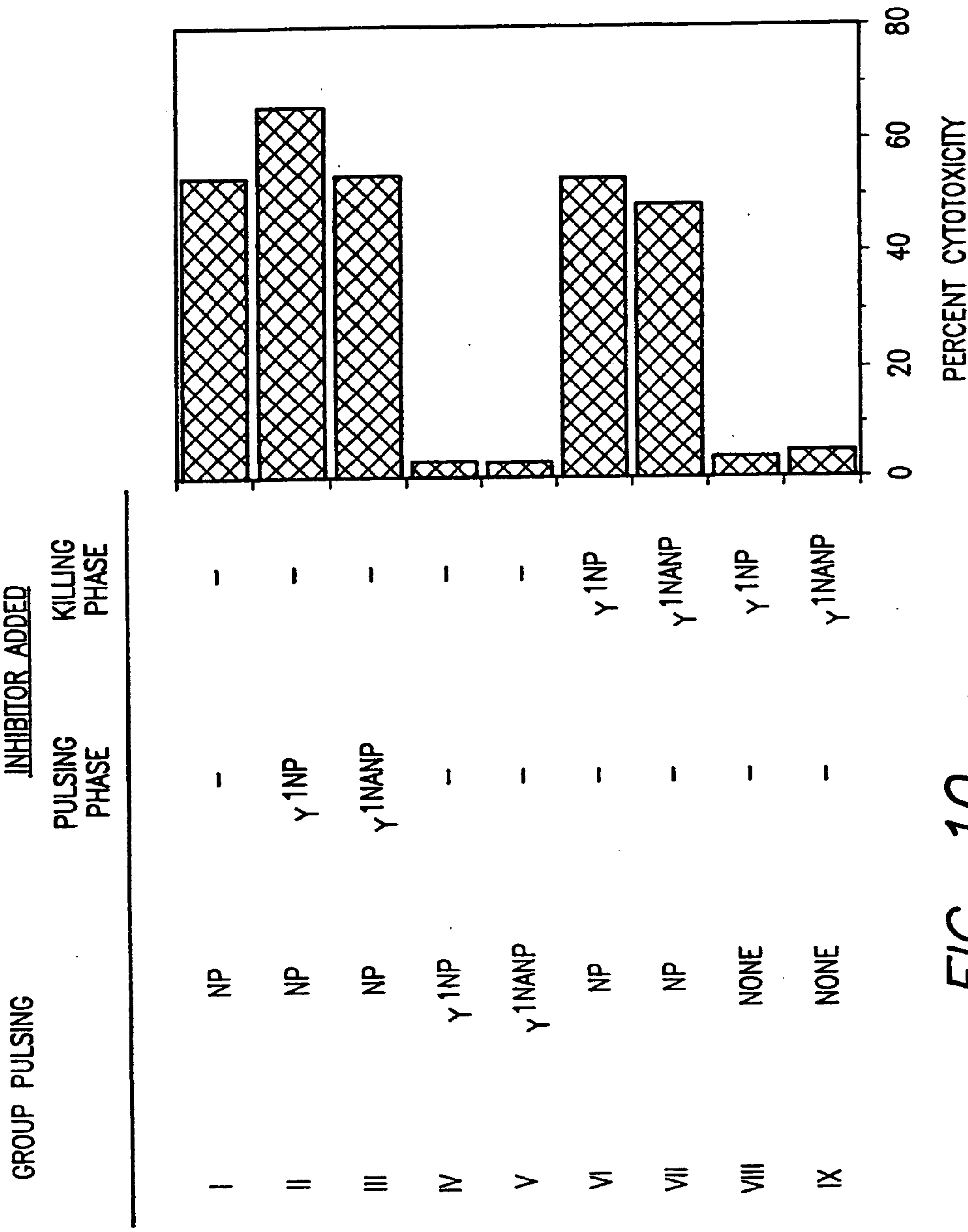
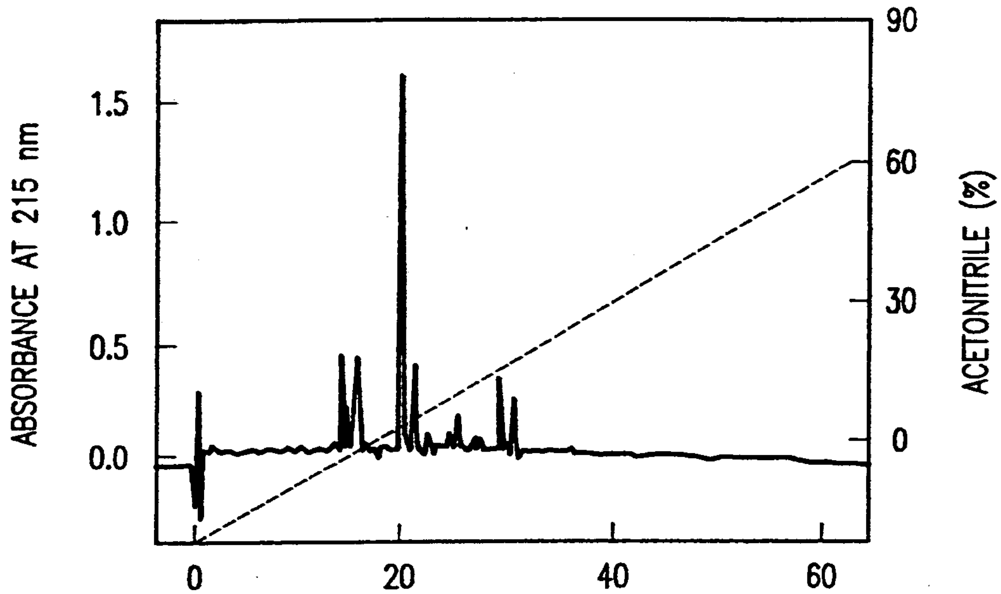

GROUP PULSING
INHIBITOR ADDED
PULSING PHASE
KILLING PHASE
I NP - -
II NP Y1NP -
III NP Y1NANP -
IV Y1NP - -
V Y1NANP - -
VI NP - Y1NP
VII NP - Y1NANP
VIII NONE - Y1NP
IX NONE - Y1NANP
0
20
40
60
80
PERCENT CYTOTOXICITY

FIG. 10

FIG. 11A



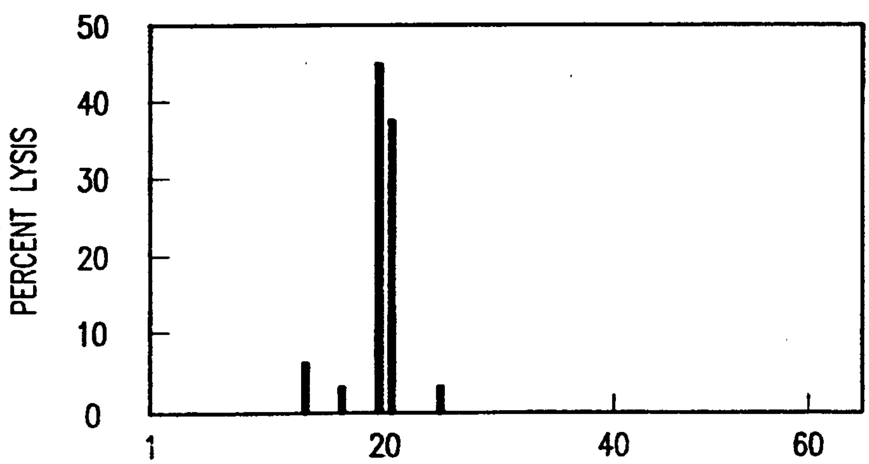
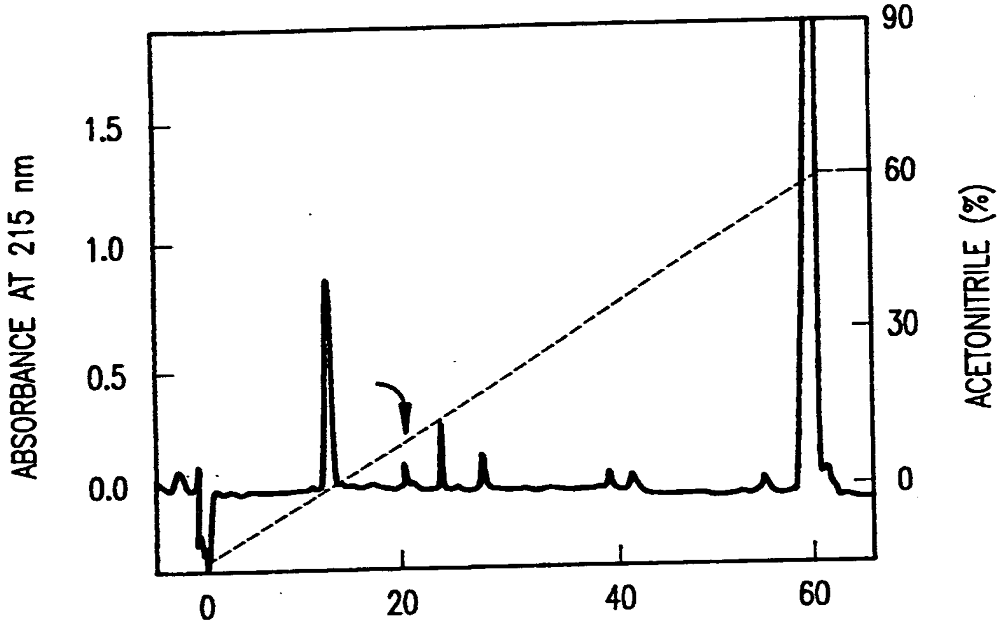


FIG. 11B

FIG. 11C



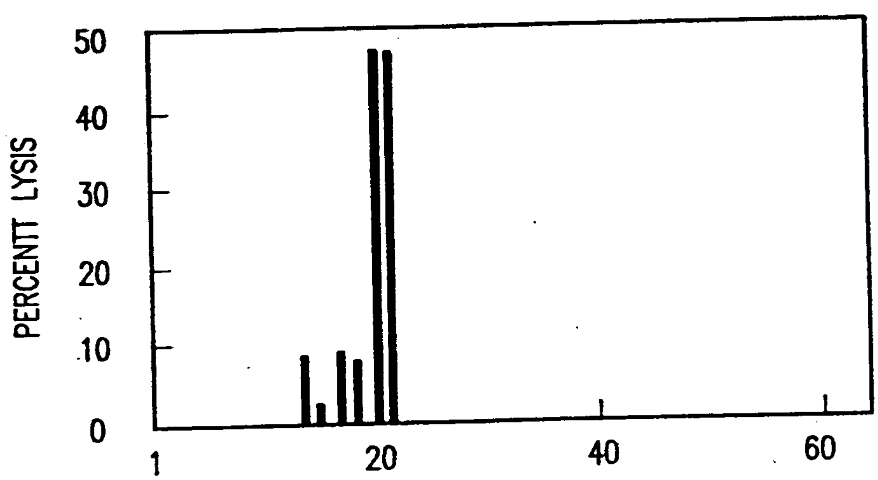
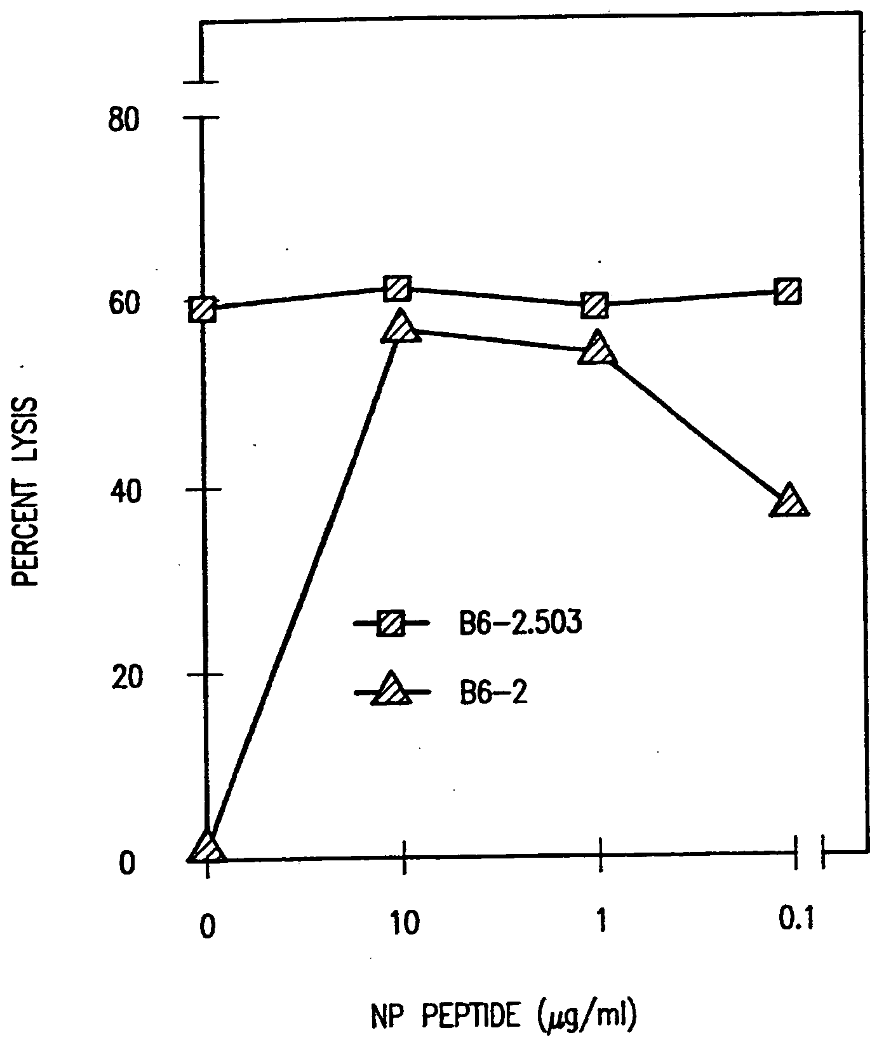


FIG. 11D

FIG. 12



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06090

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 15/28; C07H 15/00; C12N 15/00
US CL :530/387.3; 424/133.1; 536/23.53, 23.4; 435/320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.3; 424/133.1; 536/23.53, 23.4; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Experimental Medicine, Volume 165, issued June 1987, Bastin et al., "Use of synthetic peptides of influenza nucleoprotein to define epitopes recognized by Class I-Restricted cytotoxic T lymphocytes", pages 1508-1523, see Materials and Methods.	1-12
Y	Proceedings of the National Academy of Sciences USA, Volume 88, issued June 1991, Billetta et al., "Immunogenicity of an engineered internal image antibody", pages 4713-4717, see Figure 1.	1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 SEPTEMBER 1994	05 OCT 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	LILA FEISEE
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 348, issued 15 November 1990, Rotzschke et al., "Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells", pages 252-254, see entire document.	1-12
Y	Protein Engineering, Volume 4, Number 2, issued 1990, Sollazzo et al., "Expression of an exogenous peptide epitope genetically engineered in the variable domain of an immunoglobulin: implications for antibody and peptide folding", pages 215-220, see abstract.	1-12